STUDIES ON PHYSIOLOGICAL DEGRADATION PRODUCTS OF CHOLESTEROL

By

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SECTION I

GENERAL INTRODUCTION

CHEMISTRY OF STEROLS

The sterols are unsaponifiable lipids obtained from the extraction of plant or animal tissues with organic solvents. They consist of C_{27}-C_{29} secondary alcohols which are crystalline solids. In the tissues of the vertebrates, cholesterol a C_{27} alcohol of the formula $C_{27}H_{46}O$ is the principal sterol.

The early work of Wieland, Windaus, Diels, Rosenheim and King (see Fieser and Fieser, 1959, for review) established the structure of cholesterol as having a cyclopentanoperhydrophenanthrene nucleus (three fused cyclohexane rings with a terminal cyclopentane ring). As the chair form of cyclohexane is the more stable conformation, the three rings appear to be in chair forms. In the chair form of cyclohexane, two types of substituents can be recognized: those joined with an equatorial bond and those joined with axial (Hassel and Ottar, 1947; Barton, 1950).

The substituents above the plane of the ring system are called β-substituents and those below the plane of the ring are termed α-substituents. Substituents of

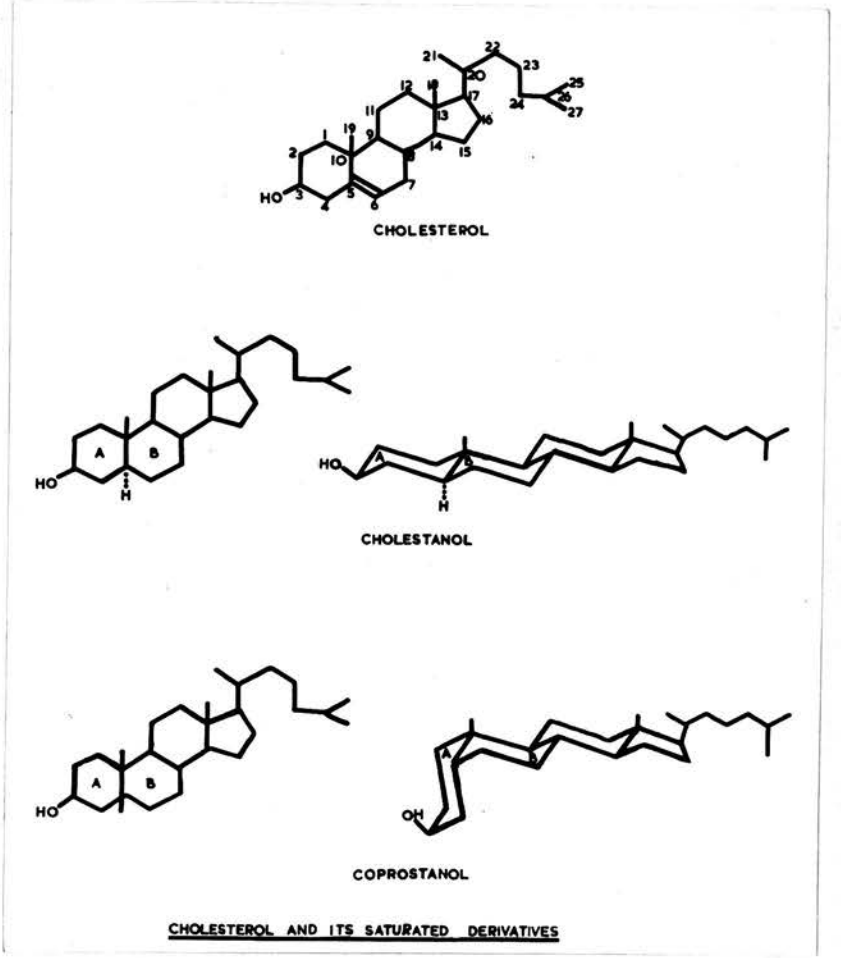
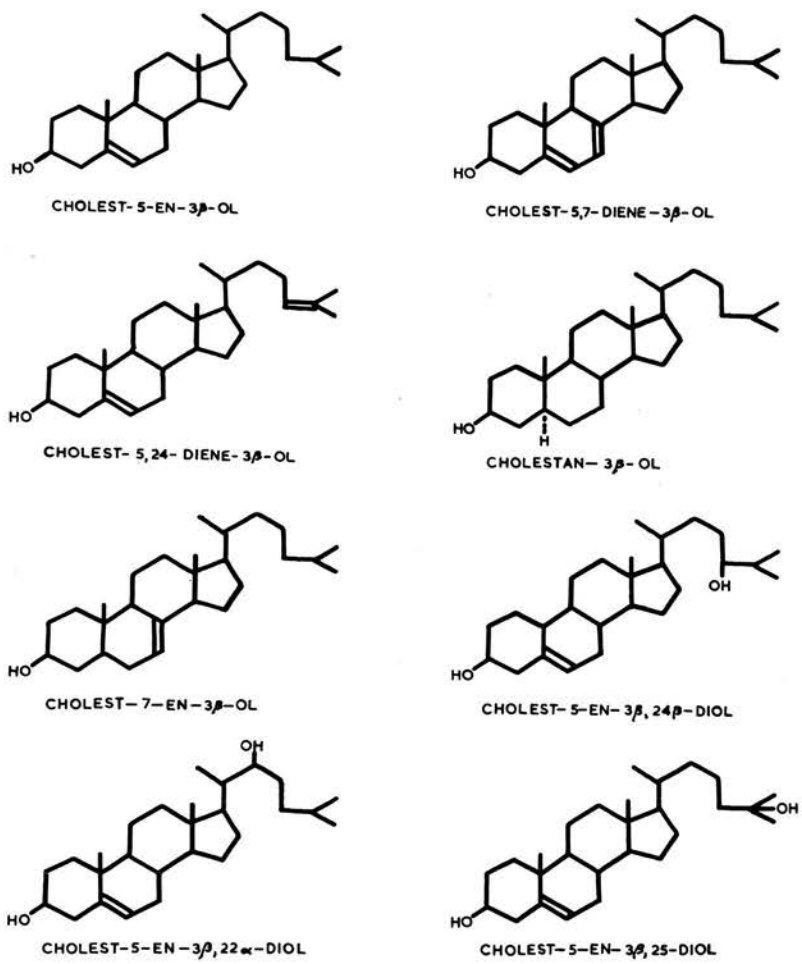

CHOLESTEROL
CHOLESTANOL
COPROSTANOL
CHOLESTEROL AND ITS SATURATED DERIVATIVES

Figure 1

unknown configurations are denoted by the Greek letter ξ . The subject of conformations of cyclohexane rings has been reviewed by Klyne (1954), Barton (1953) and Barton and Cookson (1956).

In cholesterol the cyclopentanoperhydrophenanthrene ring system has two angular methyl groups at carbon 10 and carbon 13 and an iso-octane side chain is attached in the β-position at C_{17}. The hydroxyl group is at 3β-position. A diagrammatic representation of the structure of cholesterol is given in Fig. 1.

In cholesterol there is a potential asymmetric centre at carbon 5 and the reduction of the 5, 6-double bond may lead to two compounds - one, cholestanol, having the introduced hydrogen in the α-position or trans to the angular methyl groups, and the other, coprostanol, with a 5β-hydrogen. In cholestanol the rings B and C are locked rigidly in the chair conformation by the trans fusion to rings A and D but the ring A is free to assume a different conformation. In coprostanol, since the 5-hydrogen is β, the fusion of the rings A and B is cis and the ring A assumes a different form. Nevertheless, it has the chair conformation (Fig. 1). The work on stereochemistry and nomenclature has been reviewed by Klyne (1957).



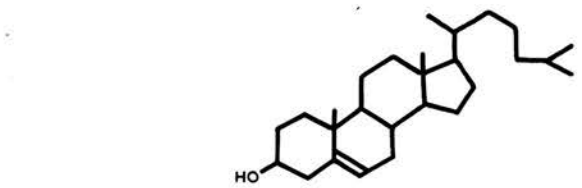
CHOLESTEROL AND 'COMPANIONS'

Figure 2

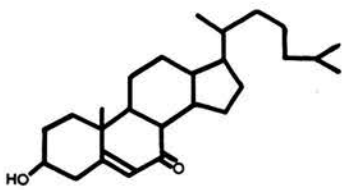
Occurrence

Cholesterol is found in all tissues both free and esterified with higher fatty acids. The sterol fractions obtained from various body tissues consist of a number of other sterols beside cholesterol. These compounds have been called 'cholesterol companions' (Fig. 2).

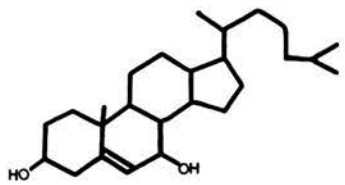
Cholest-5-en-3β-ol (cholesterol) obtained from tissue extracts has always been found to be contaminated with these compounds. Cholest-5, 7-diene-3β-ol was isolated from pig skin (Windaus, 1936). Schoenheimer indicated the presence of cholestan-3β-ol which was later isolated (Fieser, 1951) along with another companion cholest-7-en-3β-ol (Fieser, 1951; 1953; Idler and Bauman, 1952). Cholest-5, 24-diene-3β-ol was isolated from chick embryo (Stokes, Fish and Hickey, 1956) and also from rat skin (Stokes, Hickey and Fish, 1958). Cholesterol obtained from horse or human brain (Ercoli, 1953 de Frisco & de Ruggieri/) or from spinal cord preparations contains cholest-5-en-3β, 24β-diol. Another companion 22α-hydroxycholesterol was isolated from a lily (Stabursvik, 1953). Brain extracts yielded cholesterol containing cholest-5-en-3β, 25-diol and



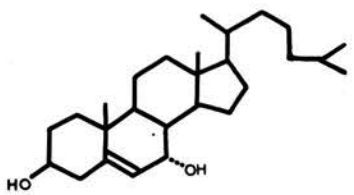
CHOLEST-5-EN-3β-OL



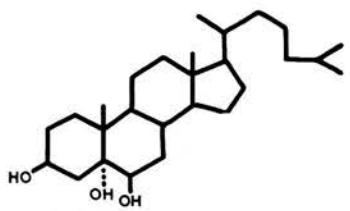
CHOLEST-5-EN-3β-OL-7-ONE



CHOLEST-5-EN-3β,7β-DIOL



CHOLEST-5-EN-3β,7α-DIOL



CHOLESTAN-3β,5α,6β-TRIOL

CHOLESTEROL AND SOME OF ITS AUTOXIDATION PRODUCTS.

Figure 3

cholestan-3β, 5α, 6β-triol but these two compounds could very well be autoxidation products of cholesterol since old samples of pure cholesterol have been shown to have got contaminated with these autoxidation products.

Autoxidation

Cholesterol is readily oxidized in colloidal solutions when exposed to excess oxygen. The chief products of autoxidation (Fig. 3) isolated are cholest-5-en-3β-ol-7-one, cholest-5-en-3β, 7β-diol and cholest-5-en-3β, 7α-diol (Bergstrom and Wintersteiner, 1941, 1942). Cholestan-3β, 5α, 6β-triol has also been isolated as an autoxidation product (Danielsson, 1960).

The mechanism of these oxidations is not known. Since the oxygen used is molecular and the position 7, allylic to the 5, 6-double bond, seems to be very sensitive to these oxidations, Bergstrom suggested that these autoxidation products may be due to the formation of a hydroperoxide at position 7 which could cleave in presence of water or metal ions in biological systems to give these products (Bergstrom, 1943).

The breakdown of hydroperoxides to keto- and hydroxy- products is a well known phenomenon

(Kornblum and De La Mare, 1951) and it is now known that cholesterol can be photo-oxygenated at the allylic position 7 to give 7α-hydroperoxides (Schenck, Gollnick and Neumuller, 1958b). It seems that in these autoxidations the molecular oxygen is trapped on to the allylic position 7 as OOH which is an unstable molecule and readily cleaves to give the 7-oxygenated compounds and their dehydration products.

However, this hydroperoxide formation does not explain the autoxidative formation of cholestan-3β, 5α,-6β-triol. Brill (1963) showed the formation of trace amounts of epoxides in his photo-sensitized oxygenation reactions showing that in presence of a catalyst molecular oxygen can be used to form an epoxide. Nickon et.al (1963) havemade this method quantitative for preparation of some keto- and hydroxy-epoxides. It is known that the reductive cleavage of epoxides gives trans-diaxial products (Fieser and Fieser, 1959). It would thus seem that the role of a 5, 6-epoxide as an intermediate in the autoxidative formation of cholestan-3β, 5α, 6β-triol can not be ruled out.

Bile Acids

Gmelin (1828) isolated from non-saponifiable portion of ox bile an acid, cholic acid. Several other bile acids have been isolated by different workers.

Bile acids are C_{24} saturated steroid, derived from coprostane, having a carboxyl group at C_{24} and hydroxyls at 3, 6, 7 and 12 positions. The positions of the hydroxyls vary in different bile acids but all natural bile acids have a hydroxyl group at 3α-position. The chemistry of bile acids has been reviewed by Fieser and Fieser (1959), Rodd (1953) and Shoppee (1958).

It is now known that bile acids are formed from cholesterol in the animal organism. Although a number of bile acids have been isolated, it is recognized that cholic acid (3α, 7α, 12α-trihydroxycholanic acid) and chenodeoxycholic acid (3α, 7α-dihydroxycholanic acid) are the main or 'primary' bile acids in mammals, formed from cholesterol. The work on the metabolism of bile acids has been reviewed by Bergstrom, Danielsson and Samuelsson (1962).

DEGRADATION

Cholesterol can be metabolized into various classes of compounds in the tissues of the body. It has been shown to be a precursor of steroid hormones, vitamin D, bile acids and neutral sterols. Quantitatively the most important pathway of degradation is the conversion to bile acids and neutral sterols.

Lifschutz (1914) was the first to suggest the possibility of formation of bile acids from cholesterol. The investigations on the structures of cholesterol and the bile acids, and the findings that a close similarity existed between the two (Fig. 4), seemed to lend support to Lifschutz's theory.

The first direct evidence that cholesterol was indeed degraded to bile acids in the animal organism was produced when labelled cholic acid was isolated from the bile of a dog to which deuterium-labelled cholesterol had been administered (Bloch, Berg and Rittenberg, 1943). Similar experiments using rabbit (Kendahl and Sjovall, 1955) and rats (Bergstrom and Norman, 1953) produced labelled bile acids from labelled cholesterol.

If, then, cholesterol is converted to cholic acid, four changes in its structure must necessarily take place.

A) Cleavage of the side-chain to give a C_{24}-terminal carboxyl group.

B) Changes associated with the steroid nucleus.

i) Addition of hydroxyl groups at 7α and 12α-positions.

ii) Saturation of the 5, 6-double bond to give a 5β-hydrogen and so a cis-fusion of the rings A and B (coprostan series).

iii) Epimerization of the 3β-hydroxyl group of cholesterol to 3α-hydroxyl of cholic acid.

The sequence in which these changes are brought about is not yet known. Most studies on the sequence of reactions involved in the degradation of cholesterol to bile acids, have been done on postulated synthetic intermediates. Various *in vivo* and *in vitro* techniques have been used but since the liver is capable of oxidizing a variety of parenterally administered C_{27}-sterols to C_{24}-acids (Hanahan and Wakil, 1953; Bergstrom, 1955), and this capacity is retained in *in vitro* preparations, it is very difficult to say that the compounds used in these systems are indeed intermediates in this degradation. Nevertheless, most studies have utilized this approach involving 'possible intermediates'.

There are two main pathways in which the above mentioned changes could be brought about; either the side-chain is cleaved before any other changes, or the hydroxylations and the other structural changes associated with the steroid nucleus will precede the oxidation of the side-chain. If the side-chain was oxidized first to give a C-24-carboxyl, 3β-hydroxy-chol-5-enic acid would be an intermediate, but if the steroid nucleus is modified before the oxidation of the side-chain 3α, 7α, 12α-trihydroxy-coprostane would be an intermediate. Bergstrom (1955) showed that the latter compound was rapidly converted into cholic acid, in a bile fistula rat, while 3β-hydroxy-chol-5-enic acid did not give cholic acid.

However, Usui and Yamasaki (1960) found that 3β-hydroxy-chol-5-enic acid was hydroxylated at position 7, but these authors did not establish the configuration at C_7 of the product formed. It is partly due to the difficulty in preparing the 7-hydroxy-chol-5-enic acid. This compound was first prepared by Haslewood (1934) by chromic acid oxidation of 3β-hydroxy-chol-5-enic acid and the subsequent reduction of the 7-ketone. The configuration at C_7 was, however, not established.

It will be of considerable interest to establish whether 3β, 7α-dihydroxy-chol-5-enic acid is converted to bile acids in say a bile fistula rat.

But, if in view of Bergstrom's experiments it is assumed that the steroid nucleus is modified before the complete oxidation of the side-chain, then there are several possibilities.

i) That the 3β-hydroxyl of cholesterol epimerizes to 3α-hydroxyl of cholic acid before other changes.

ii) That the saturation of the 5, 6-double bond precedes other structural changes.

Or iii) That hydroxylations at C_7 and C_{12} occur before other changes.

The saturation of the 5, 6-double bond may occur in situ or it may isomerize to position 4 and 5 before saturation. Chemically the latter path would be more suitable because it is known that reduction of 4, 5-double bond gives more of the 5β-hydrogen, that is compounds of the coprostan series (Grasshof, 1934; Barton, 1949), while the reduction of 5, 6-double bond gives compounds of the cholestane series (Hershberg et. al) 1951; Nace, 1951).

The epimerization of the 3β-hydroxyl may proceed through a 3-keto derivative. If this was so, the H at 3α and the H-atom of the OH-group would be lost during the course of the reaction. Also the 3-keto group would be expected to shift the double bond from 5, 6 into the stable position at 4, 5. In fact, Samuelsson (1963) has shown that cholesterol labelled with tritium at position 3-alpha lost its tritium during the course of its conversion to bile acids which lends support to the contention that the epimerization proceeds through a 3-keto intermediate.

If the epimerization of the 3-hydroxyl preceded the other reactions, then cholest-5-en-3-one, cholest-5-en-3α-ol, cholest-4-en-3-one or cholest-4-en-3α-ol would be expected as intermediates while if the saturation preceded other reactions, coprostan-3β-ol, coprostan-3-one and coprostan-3α-ol would be intermediates.

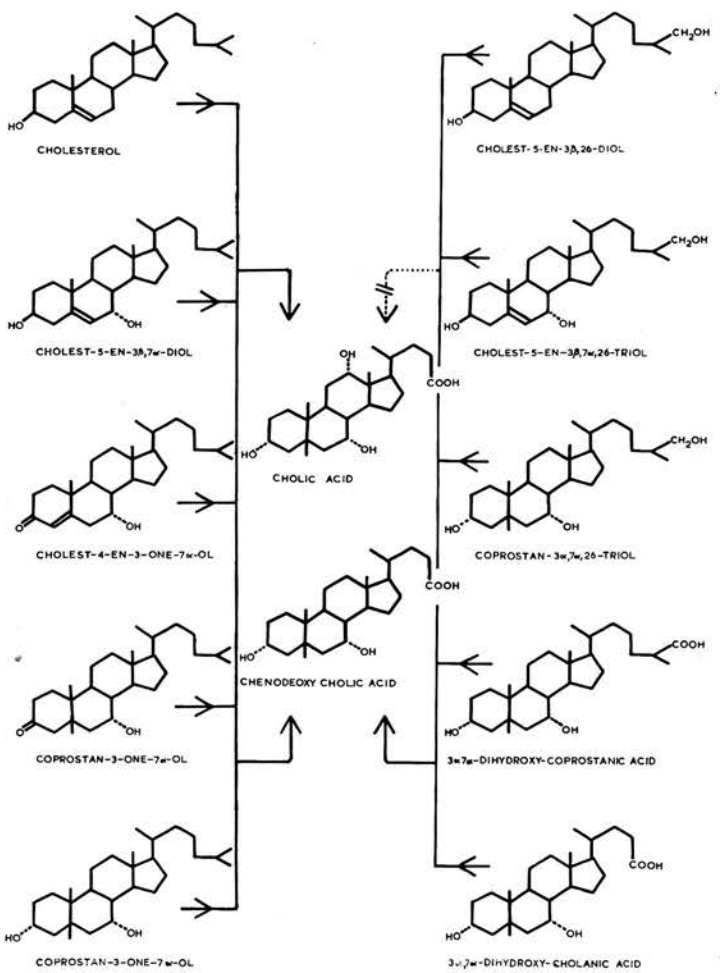
Harold, Jayko and Chaikoff (1955) and Harold et al. (1957) used a number of these saturated and unsaturated compounds with an oxygen function at carbon-3 in their bile fistula rats and found that none of them was converted into cholic acid. They concluded that the hydroxylations at C_7 and/or C_{12} must precede the inversion of the 3-hydroxyl.

Since the reduction of 4, 5 double bond leads to compounds of the coprostane series it would be of interest to test cholest-4-en-3α-ol and coprostan-3-one in these experiments.

7α-Hydroxylation

As an extension of Harold and Chaikoff's studies Bergstrom and Lindstedt (1956) and Lindstedt (1957a) used labelled cholest-5-en-3β, 7α-diol and coprostan-3α, 7α-diol in their experiments with bile fistula rat and showed that both were converted into cholic and chenodeoxycholic acids. On the basis of these experiments Bergstrom concluded that 7α-hydroxylation is an early and essential step in the conversion of cholesterol to bile acids.

As pointed out earlier, the position 7 is very reactive and so it is very likely that the first step towards the degradation of cholesterol to bile acids is an attack at position 7. The complete mechanism of this hydroxylation is not known and the enzyme or enzymes catalysing this reaction have not been purified.



COMPOUNDS SHOWN TO BE POSSIBLE INTERMEDIATES IN THE FORMATION OF CHOLIC AND CHENODEOXY CHOLIC ACIDS.

Figure 4

The subject will be discussed in a greater detail later (p. 31). Again because of the sensitivity of this position to autoxidation, studies on this initial hydroxylation of the cholesterol molecule at C_7 have been difficult.

Cholesterol (Bloch et al. 1943; Bergstrom and Norman, 1953; Kendahl and Sjovall, 1955) cholest-5-en-3β, 7α-diol and coprostan-3α, 7α-diol (Bergstrom and Lindstedt, 1956; Lindstedt, 1957a) cholest-4-en-3-one-7α-ol, coprostan-3-one-7α-ol (Danielsson, 1961a and b) have been shown to convert into cholic and chenodeoxycholic acids while compounds with some modifications in the side-chain like cholest-5-en-3β, 26-diol, cholest-5-en-3β, 7α, 26-triol (Danielsson, 1961d), coprostan-3α, 7α, 26-triol (Berseus and Danielsson, 1963), 3α, 7α-dihydroxy coprostanic acid (Bridgewater and Lindstedt, 1957) or 3α, 7α-dihydroxy-cholanic acid (Lindstedt and Sjovall, 1957) all have been shown to convert into chenodeoxycholic acid and very insignificant amounts of cholic acid (Fig. 4). These studies show that 12α-hydroxylation does not occur once the oxidation of the side-chain has started.

Danielsson (1961e) studied the metabolism of cholest-5-en-3β, 12α-diol in the bile fistula rat and found that it was converted in part to cholic acid, but he pointed out that this could be due to 7α-hydroxylation of deoxycholic acid which could easily have formed from the administered cholest-5-en-3β, 12α-diol. To establish the direct formation of cholic acid from 12α-hydroxycholesterol the rabbit would be a suitable animal since in this species deoxycholic acid can not be hydroxylated at position 7 (Lindstedt and Sjovall, 1957; Bergstrom et al. 1955).

From the evidence accumulated above that 3,7-dihydroxy compounds, saturated and unsaturated, are converted to cholic acid while 12α-hydroxycholesterol cannot be considered as a direct precursor of cholic acid, it might be reasonable to assume that in the sequence of hydroxylations of the nucleus 7α-hydroxylation precedes the 12α-hydroxylation and that the 12α-hydroxylation precedes any modifications of the side-chain and also that 7α-hydroxylation can occur even after the initiation of the oxidation of the side-chain.

Inversion of Configuration at Position 3 and Saturation of the Double Bond

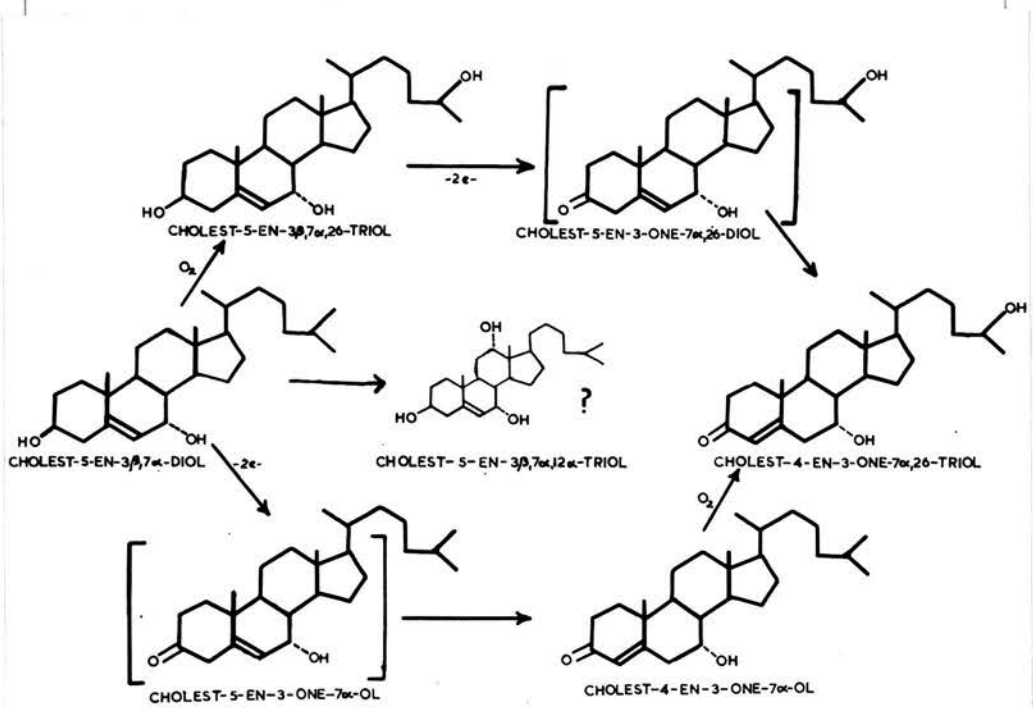
From the above discussion it seems that hydroxylation at position 7 occurs before the inversion of the 3β-hydroxyl group and the saturation of the double bond and also before the introduction of 12α-hydroxyl group. Coprostan-3α, 7α-diol has been shown to be on the pathway of degradation of cholesterol to cholic and chenodeoxycholic acids (Lindstedt, 1957a). Thus, if 7α-hydroxylation occurs before 12α-hydroxylation and the inversion of the 3β-hydroxyl, and also if the inversion takes place via 3-keto derivatives as suggested by Yamasaki et al. (1959a) on the basis of their in vitro studies (see also p. 11) then cholest-4-en-3-one-7α-ol and coprostan-3-one-7α-ol can be postulated as intermediates. If the 3β-hydroxyl is oxidized to a ketone after the introduction of the 7α-hydroxyl, the 5, 6-double bond would be expected to isomerize into 4, 5-position almost simultaneously and the possibility of cholest-5-en-3-one-7α-ol as a stable intermediate is remote. Samuelsson's studies with 3α-^{3}H-cholesterol (p. 11) and his suggestion (Samuelsson, 1959) that the saturation

of the 5, 6-double bond in the conversion of cholesterol to chenodeoxycholic acid in pig is a stereospecific cis-addition from the β-side of the molecule lend support to Yamasaki's suggestion (also Green and Samuelsson, 1964).

Danielsson (1961 a and b) showed that cholest-4-en-3-one-7α-ol and coprostan-3-one-7α-ol were indeed converted into both cholic and chenodeoxycholic acids in the bile fistula rat.

In vitro studies with cholest-5-en-3β, 7α-diol using mouse liver homogenates have shown that cholest-4-en-3-one-7α-ol was one of the metabolites (Danielsson, 1961c). In the same experiments two other metabolites were obtained one of which because of its spectral and chromatographic properties was suggested to be cholest-4-en-3-one-7α, 26-diol and the other was identified as cholest-5-en-3β, 7α, 26-triol. The same triol was also obtained when cholest-5-en-3β, 26-diol was used as the substrate (Danielsson, 1961a).

Identical results have been obtained in rat liver in vitro preparations (Boyd and Hutton, 1964). Using cholest-5-en-3β, 7α-diol as the substrate in rat liver microsomal preparations, cholest-4-en-3-one-7α-ol was



POSSIBLE PATHWAYS OF DEGRADATION OF CHOLEST-5-EN-3β,7α-DIOL

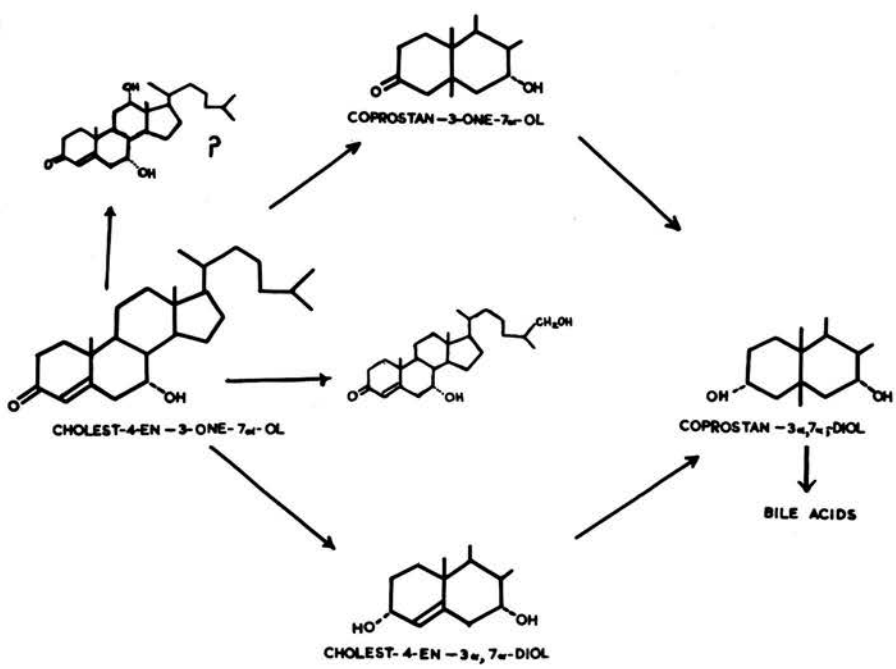
Figure 5

isolated as a product. Two other products were obtained, one of them identified as cholest-5-en-3β, 7α, -26-triol and the other suggested to be cholest-4-en-3-one-7α, 26-diol (Fig. 5).

If cholest-4-en-3-one-7α-ol was metabolized to coprostan-3α, 7α-diol or chenodeoxycholic acid, the 4, 5-double bond and the 3-ketone have to be reduced and depending upon which of the two is reduced first, cholest-4-en-3α, 7α-diol and coprostan-3-one-7α-ol would be intermediates.

Coprostan-3-one-7α-ol has been shown to convert into cholic and chenodeoxycholic acids in the bile fistula rat (Danielsson, 1961d). Boyd and Hutton (1964) isolated this compound when cholest-4-en-3-one-7α-ol was used in their rat-liver in vitro preparations. Coprostan-3α, 7α-diol, cholest-4-en-3α, 7α-diol and a more polar compound suggested to be cholest-4-en-3-one-7α-26-diol were also obtained.

Cholest-4-en-3α, 7α-diol would be expected to give 5β-hydrogen on saturation by chemical catalytic reduction, but in in vitro systems this was found not to



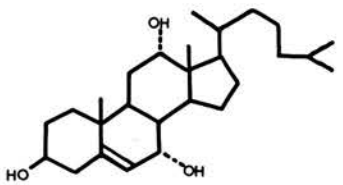
POSSIBLE PATHWAYS OF DEGRADATION OF CHOLEST-4-EN-3-ONE-7α-OL

Figure 6

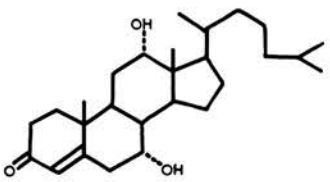
convert into coprostan-3α, 7α-diol as readily as coprostan-3-one-7α-ol (Boyd and Hutton, 1964). Thus, it seems that the preferred path of epimerization of 3β-hydroxyl and the saturation of the double bond of cholest-5-en-3β, 7α-diol is through cholest-4-en-3-one-7α-ol and the corresponding saturated compound coprostan-3-one-7α-ol which on reduction gives coprostan-3α, 7α-diol. All these compounds have been tested as precursors in the bile fistula rat and have also been isolated in in vitro systems (Fig. 6).

12α-Hydroxylation

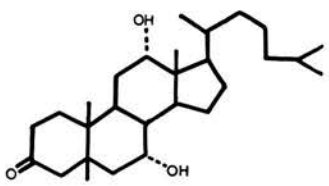
Up to date no compounds carrying a 12α-hydroxyl group have been isolated from incubations of cholesterol or other C_{27} neutral sterols not already carrying a 12α-hydroxyl. Consequently we do not know where on the pathway of degradation the 12α-hydroxylation occurs. On the evidence cited above it is clear that the 12α-hydroxylation does not occur before 7α-hydroxylation nor after the initiation of the oxidation of the side-chain. Thus it could be considered to occur either before the epimerization of the 3β-hydroxyl and saturation of the double bond or after it. In the former case, cholest-



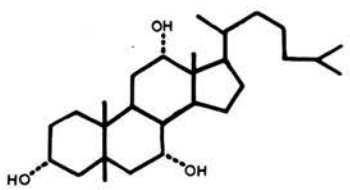
CHOLEST-5-EN-3β,7α,12α-TRIOL



CHOLEST-4-EN-3-ONE-7α,12α-DIOL



COPROSTAN-3-ONE-7α,12α-DIOL



COPROSTAN-3α,7α,12α-TRIOL

SOME POSSIBLE 12α-HYDROXYLATED INTERMEDIATES IN THE FORMATION OF CHOLIC ACID

Figure 7

5-en-3β, 7α, 12α-triol would be an intermediate while in the latter case coprostan-3α, 7α, 12α-triol would be an intermediate. This latter compound has indeed been shown to convert into cholic acid (p. 9), while the former has not yet been synthesized to test in bile fistula animals, nor has its formation ever been indicated in any in vitro studies.

If cholest-5-en-3β, 7α, 12α-triol was found to be an intermediate and the epimerization and saturation proceeded by the same series of reactions as for cholest-5-en-3β, 7α-diol, then cholest-4-en-3-one-7α, -12α-diol, coprostan-3-one-7α, 12α-diol and coprostan-3α, 7α, 12α-triol could be postulated as intermediates.

If cholest-5-en-3β, 7α, 12α-triol is found not to be an intermediate, then 12α-hydroxylation may occur after the initiation of the set of reactions involved with the inversion at C_3 and the saturation of the double bond. In either case the above mentioned compounds would be intermediates (Fig. 7).

The fact that cholest-5-en-3β, 7α-diol, cholest-4-en-3-one-7α-ol, coprostan-3-one-7α-ol and coprostan-3α, 7α-diol are all converted into cholic acid in the bile fistula rat (p. 13), shows that a 12α-hydroxylation

can occur after any of these stages and thus supports the hypothesis that the above mentioned compounds may be intermediates.

Because of the marked difference in reactivity of the hydrogen at C_7 compared with C_{12} of the cholesterol molecule, hydroxylation reaction mechanisms at the two centres may differ considerably. Hydroxylation at C_7 is easy and thus a number of 7α-hydroxylated intermediates have been synthesized by a direct introduction of the oxygen function. The C_{12}, on the other hand, being saturated and sterically hindered, is difficult to attack and that is why as a rule the syntheses of 12α-hydroxylated compounds start with a compound already carrying a 12α-hydroxyl group, namely deoxycholic acid (see also p. 31).

Structural Changes in the Side-Chain

As discussed in the preceding pages the structural changes on the steroid nucleus seem to precede the complete oxidation of the side-chain. The cleavage of the side-chain may proceed by:

(i) a direct removal of the isopropyl group, a reaction analogous to the cleavage of the cholesterol side-chain by adrenal enzymes to yield pregnenolone and isocaproic aldehyde (Lynn et al. 1955; Staple et al. 1956), or (ii) a gradual oxidation of the three terminal carbons, or (iii) an initial oxidation at one of the terminal (C_{25}, C_{26} or C_{27}) carbons facilitating an attack at C_{24} leading to β-oxidation at C_{24}.

In the first case 24- or 25-hydroxy, 24, 25-dihydroxy and 24-dehydro derivatives would be expected intermediates while in the latter two cases the initial attack would be at one of the terminal carbons to give 26- or 27-hydroxyl and carboxyl derivatives followed by a number of other oxygenated and dehydrated derivatives.

Since the most stable conformation of the side-chain may be a straight chain structure (Fieser and Fieser, 1959), the terminal methyls at C_{26} and C_{27} are the most easily accessible of all the carbon atoms of the side-chain. Thus it would seem that the initial attack on the side-chain may be an omega-oxidation at one of the terminal methyl carbons. This may be followed by other oxygenations and dehydrations to give a C_{24} structure.

Studies on the mechanism of the cleavage of the side-chain have indicated that an omega-oxidation is probably the first step in this degradation; a number of 26-hydroxylated compounds have been isolated as intermediates in in vitro experiments and a number of 26-oxygenated C_{27} steroids have been shown to be precursors of bile acids in vivo.

Evidence for Omega-Oxidation (26-Hydroxylation)

Using cholesterol labelled at C_{26} with C^{14}, it has been shown that rat-liver slices (Meier et al. 1952) or cell free preparations of mouse liver (Horning et al. 1957) can oxidize it to C^{14}-labelled CO_2. Whitehouse et al. (1959) have also shown that mouse liver mitochondria catalyse the oxidation of the terminal methyl group of cholesterol to CO_2.

In this formation of CO_2, 26-hydroxy, 26-carboxy, 25-hydroxy and 25-dehydro compounds would be postulated as intermediates. Fredrickson (1956) in his in vitro studies with labelled cholesterol identified as products 26- and 25-hydroxylated derivatives along with some unidentified acidic products. Labelled CO_2 was also evolved. Danielsson (1961d) using mouse liver

homogenates identified 26-hydroxycholesterol as a metabolite of cholesterol. He also found that mouse liver mitochondria were much more active for this reaction than were rat liver mitochondria.

Gurin and coworkers (Lynn et al. 1955; Whitehouse et al. 1959) used a liver mitochondrial preparation which they called 'cholesterol oxidase' system and reported the conversion of 26-C^{14}-cholesterol to 25-dehydrocholesterol (cholest-5, 25-diene-3β-ol).

Since the use of cholesterol as the substrate in in vitro systems also leads to a number of autoxidation products it is considered advantageous to perform the in vitro studies on the possible intermediates rather than cholesterol itself. Danielsson (1961c) studied the metabolism of cholest-5-en-3β, 7α-diol in mouse liver homogenate and identified the 26-hydroxylated derivative, cholest-5-en-3β, 7α, 26-triol as a metabolite along with cholest-4-en-3-one-7α-ol. In the same system he also found another metabolite which was suggested to be the 26-hydroxylated derivative of cholest-4-en-3-one-7α-ol. These two 26-hydroxylated compounds were also formed when cholest-5-en-3β, 7α-diol was incubated with

mitochondria plus the 'supernatant fraction' of rat liver homogenate (Boyd and Hutton, 1964). Berseus and Danielsson (1963) have reported 26-hydroxylation of coprostan-3α, 7α-diol.

Enomoto (1962) has shown the conversion of coprostan-3α, 7α, 12α-triol to 3α, 7α, 12α-trihydroxy-coprostanic acid, the main bile acid from cholesterol in the toad, to proceed through coprostan-3α, 7α, 12α, 26-tetrol.

Staple and Gurin (1962) found that their 'cholesterol oxidase' system can oxidize coprostan-3α, 7α, 12α-triol labelled at C_{27} with C^{14} to coprostan-3α, 7α, 12α, 27-tetrol.

The same results were obtained in mouse and rat liver homogenates (Danielsson, 1960). Small amounts of 3α, 7α, 12α-trihydroxycoprostanic acid were also obtained. Suld et al. (1962) confirmed the findings of the above two groups of workers and, in addition, isolated labelled propionic acid from the side-chain. Propionic would result from the cleavage of the C_{24}-C_{25} bond of 3α, 7α, 12α-trihydroxycoprostanic acid. Staple

and Rabinowitz (1962) have isolated labelled 3α, 7α, 12α-trihydroxycoprostanic acid from human subjects given labelled cholesterol.

All the above mentioned experiments on the mechanism of the cleavage of the side-chain have shown an initial attack on C_{26} giving 26-hydroxylated and carboxy intermediates. A number of these 26-hydroxy compounds have also been shown as precursors of bile acids in bile fistula animals (p. 13).

Evidence for β-Oxidation (24-Hydroxylation)

Whitehouse et al. (1960, 1961) incubated cholesterol-26-C^{14} with liver mitochondrial preparations and under certain conditions could isolate labelled acetone. Using 26-C^{14}-cholesterol and 25-C^{14}-cholesterol it was shown that in both cases the acetone formed was labelled indicating that the formation of acetone was due to a direct cleavage of the C_{24}-C_{25} bond.

If acetone formation was due to a pathway of degradation of the side-chain 24- and 25-hydroxy-cholesterols, 24, 25-dihydroxycholesterol and 24-dehydrocholesterol are possible intermediates.

Fredrickson (1956) isolated 25- and 26-hydroxy-cholesterols from cholesterol incubations with mitochondrial systems of Anfinsen and Horning (1953) but when cholest-5-en-3β, 25-diol was tested on bile fistula rat, it did not yield cholic acid (Fredrickson and Ono, 1956).

It will be interesting to do the same studies with cholest-5-en-3β, 24-diol.

Further Evidence for Omega-Oxidation

Studies with cholest-5, 24-diene-3β-ol have shown that this compound could be regarded as a direct precursor of bile acids. Bile acid production in Triparanol (MER-29) treated animals does not differ markedly from untreated animals (Goodman et al. 1962a; Blohm et al. 1960). Since Triparanol is considered to block the enzyme catalysing the hydrogenation of the side-chain double bond (C_{24}-C_{25}) and so stop the biosynthesis of cholesterol (Avigan et al. 1960), these experiments indicate that desmosterol (cholest-5, 24-diene-3β-ol) might be a direct precursor of bile acids.

In vitro studies with desmosterol using rat liver mitochondria (Kritchevsky and Staple, 1962) have shown that the side-chain may be oxidized to produce CO_2. Goodman et al. (1962b) have confirmed these results using mouse liver mitochondria (also Danielsson and Johansson, 1964).

Shimizu et al. (1959) found that coprostan-3α, 7α, -12α-triol, coprostan-3α, 7α, 12α-triol-24-one and coprostan-3α, 7α, 12α, 24-tetrol were all converted to cholic acid in the bile fistula rat, but the rate of production of cholic acid was much slower with the latter two compounds. Whitehouse et al. (1961b) have obtained essentially the same results in their in vitro studies with rat liver mitochondrial systems. The yield of CO_2 from coprostan-3α, 7α, 12α-triol was much higher than from C_{24}-substituted compounds. But in the case of mouse, monkey and human liver mitochondria the case was reversed; the C_{24}-substituted compounds coprostan-3α, 7α, 12α, 24-one and coprostan-3α, 7α, 12α, -24-tetrol were oxidized more readily than the coprostan-3α, 7α, 12-triol. However, the same group of workers (Staple and Rabinowitz, 1962) have isolated 3α, 7α, 12α-trihydroxycoprostanic acid from the bile of a human subject given labelled cholesterol.

All these experiments have been done on 3α, 7α, 12α-trihydroxy compounds. It would be interesting to repeat such experiments with 3α, 7α-dihydroxy-coprostanic compounds with oxygen functions at C_{24} and without them, and see if these compounds are converted to chenodeoxycholic acid.

On the basis of the above information on the oxidation of the side-chain, acetone formation may not be a major pathway in the degradation of the side-chain. There are a number of experiments to show CO_2 formation and C_{26}-hydroxylation and also the ready conversion of 26-oxidized compounds into bile acids while there is not enough evidence to prove ready conversion of C_{24}-or C_{25}-hydroxylated compounds to bile acids. The acetone formation seems, at most, due to a minor pathway of degradation of the cholesterol side-chain. But it would be very interesting to investigate further this pathway.

Thus, it seems that the first step in the degradation of the side-chain is an omega-oxidation at the terminal methyl group, followed by a β-oxidation to give the C_{24}-carboxyl of the bile acids. If an omega-oxidation was

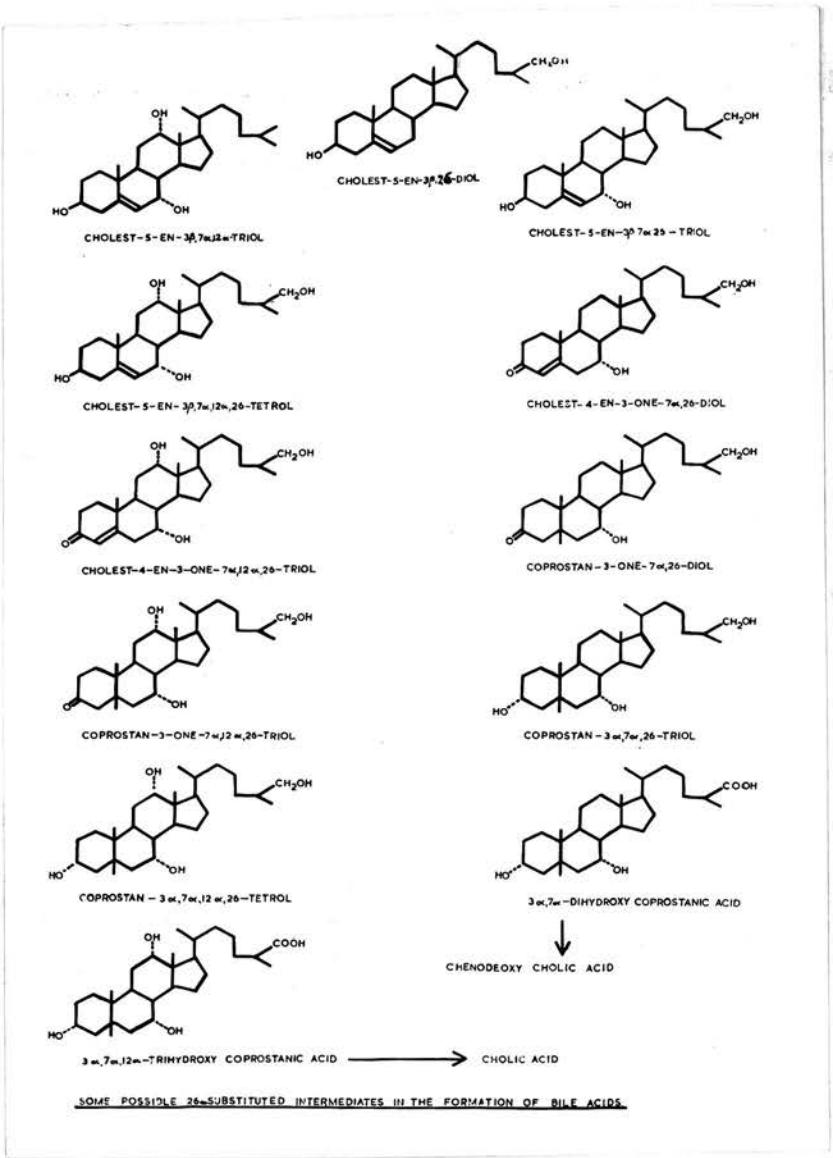

CHOLEST-5-EN-3β,26-DIOL
CHOLEST-5-EN-3β,7α,12α-TRIOL
CHOLEST-5-EN-3β,7α,26-TRIOL
CHOLEST-5-EN-3β,7α,12α,26-TETROL
CHOLEST-4-EN-3-ONE-7α,26-DIOL
CHOLEST-4-EN-3-ONE-7α,12α,26-TRIOL
COPROSTAN-3-ONE-7α,26-DIOL
COPROSTAN-3-ONE-7α,12α,26-TRIOL
COPROSTAN-3α,7α,26-TRIOL
COPROSTAN-3α,7α,12α,26-TETROL
3α,7α-DIHYDROXY COPROSTANIC ACID
CHENODEOXY CHOLIC ACID
3α,7α,12α-TRIHYDROXY COPROSTANIC ACID
CHOLIC ACID
SOME POSSIBLE 26-SUBSTITUTED INTERMEDIATES IN THE FORMATION OF BILE ACIDS.

Figure 8

the first step of this cleavage of the side-chain, depending upon at what stage of the reactions involved with the modification of the cholesterol nucleus this oxidation is brought about, a number of 26-hydroxyl and 26-carboxyl derivatives can be postulated as intermediates (see Fig. 8). A number of these compounds have already been synthesized and tested as precursors of bile acids (see pp. 13, 16) but there are a number of others which have not yet been synthesized and so not yet tested.

If the 26-hydroxylation occurs before 12α-hydroxylation, the final product will probably be chenodeoxycholic acid (since 12α-hydroxylation does not occur after 26-hydroxylation) and thus cholest-5-en-3β, 7α, 26-triol, cholest-4-en-3-one-7α, 26-diol, coprostan-3-one-7α, 26-diol, and coprostan-3α, 7α, 26-triol would be the possible hydroxylated intermediates while, if the 26-hydroxylation occurs after the introduction of the 12α-hydroxyl, cholest-5-en-3β, 7α, 12α, 26-tetrol, cholest-4-en-3-one-7α, 12α, 26-triol, coprostan-3-one-7α, 12α,-26-triol and coprostan-3α, 7α, 12α, 26-tetrol would be intermediates. It cannot be said at what stage of the modification of the nucleus the side-chain is attacked.

If a complete oxidation of the side-chain occurs during the nuclear changes, then a number of corresponding 24-carbon C_{24}-carboxylated compounds would be intermediates. If 3β-hydroxy-chol-5-enic acid can be hydroxylated at C_7 (Usui and Yamasaki, 1960) then the inversion at C_3 and saturation of the double bond would occur after this and thus a number of 24-carbon C_{24}-carboxylated compounds would be intermediates.

Danielsson and Johansson (1964) have shown that desmosterol (cholest-5, 24-diene-3β-ol), when incubated with mouse liver in vitro preparations, gave a more polar compound which was suggested to be the 26-hydroxylated derivative of desmosterol. If desmosterol was on the route of biosynthesis of bile acids from cholesterol there is a possibility of a 25-hydroxylated derivative being an intermediate which would give desmosterol on dehydration (Dauben and Bradlow, 1950; Ryer et al. 1950). If desmosterol is an intermediate and also if 25-hydroxylation is a step in the degradation, then a number of 25-hydroxylated and 24-dehydro derivatives could be postulated (see also p. 39) as intermediates.

HYDROXYLATIONS OF CHOLESTEROL MOLECULE

In the metabolism of cholesterol it has been shown that the cholesterol molecule is not completely oxidized to CO_2 but undergoes enzymic reactions which degrade it to a C_{24} structure, the bile acids, which are excreted as the end product of cholesterol degradation. As discussed in the preceding pages, this degradation is brought about by a sequence of reactions involving oxidations, dehydrations and reductions and possibly proceeds through hydroxylated and other intermediates. The positions of the cholesterol molecule which are affected in this degradation are the carbons 3, 6, 7, 12, 24, possibly 25, and 26.

The mechanism of these hydroxylations is not known. While all the carbons affected in these hydroxylations are saturated, the position 7 is unique in being allylic to the 5, 6-double bond. Because of this difference in the chemistry of the position 7 from others, there may be different mechanisms for these two types of hydroxylation and so in the animal organism two different types of enzyme systems involved.

For a number of steroid nucleus and side-chain hydroxylations it is known that the oxygen used is in a molecular state (for references see reviews by Talalay (1957) and Tamm (1962)) and that a reducing enzyme system is required (Brownie and Grant, 1956; Grant and Brownie, 1956). Using cholesterol labelled in the 7α- and 7β-positions it has been shown that the 7α-hydroxylation is a stereospecific reaction where the 7α-hydrogen is replaced by a hydroxyl without an inversion of configuration; the 7α-tritium was lost during the course of the degradation of cholesterol to cholic acid while when cholesterol-7β-H^3 was used, the cholic acid obtained was labelled (Bergstrom et al. 1958). The 11α-hydroxylation is also the same type of reaction, the hydroxyl replacing the hydrogen stereospecifically (Corey et al. 1958; Hayano et al. 1958). There is evidence that enzymatic hydroxylations at a saturated carbon atom of a steroid proceed by direct replacement of the hydrogen and not by hydration of olefinic intermediates (Hayano and Dorfman, 1954).

Discussing the 'mixed function oxidation' systems Mason (1958) proposed a scheme for the enzymatic oxygenations. He suggests that the oxygen is taken up by an enzyme forming the hydroxylating intermediate, the enzyme-oxygen complex, which supplies oxygen to the substrate. A number of oxidizing enzymes are known which can supply molecular oxygen directly to the substrate (Hayaishi, 1956).

The combination of molecular oxygen with olefines is a well known phenomenon but these reactions are usually of a free-radical type and involve chain mechanisms, but when the reactions are conducted photochemically in dilute solutions in presence of a sensitizing agent, chain reactions do not seem to occur. (The subject has been reviewed by Walling, 1957; Bergman and McLean, 1941; Schenck, 1952.)
It has been suggested (Schenck, 1957; Livingstone and Owens, 1956; Oster et al. 1959; Nickon and Bagli, 1961) that for mechanistic discussion the photo-sensitized oxygenation reactions may be arbitrarily divided into several stages. With the absorption of

light energy the substrate and the sensitizer molecules are converted into the activated states; the sensitizer then forms a sensitizer-oxygen complex which in turn supplies oxygen to the activated centre of the substrate. It has been shown for a number of olefines (Schenck et al. 1953; Schenck, 1957; Nickon and Bagli, 1961) that this is a stereospecific replacement reaction, the introduced hydroperoxide group replacing the removed hydrogen. The attack in all the known cases of steroid photo-oxygenations has been from the rear side of the molecule, presumably because of the bulky stereo-chemistry on the β-side.

In view of the known stereospecific mechanism of photo-oxygenation reactions where a sensitizing agent could form a sensitizer-oxygen complex and supply oxygen to the activated substrate there seems to be a marked similarity between the mechanism of photo-chemical oxidations and the biological oxidations. If in the latter oxidations the oxygen is trapped on to the enzyme as O_2 (O O) and is then supplied to the activated centre of the substrate, there is a possibility of the intermediate formation of a steroid-hydroperoxide

which being unstable is cleaved at once in the presence of some reducing enzymes.

The position 7 being the most reactive of all it would be possible that the first attack on the steroid molecule is at C_7 to form a 7α-hydroperoxide. This mechanism could reasonably explain the ready hydroxylation at C_7, which is most easily activated because of the allylic double bond, but it does not explain the hydroxylations at saturated centres which could only form a hydroperoxide if there is a system existing in the animal organism which could activate the substrate in these specific positions.

However, it is known that saturated hydrocarbons can autoxidize in air to form hydroperoxides at saturated centres also. This property has been shown for straight chain as well as cyclic saturated hydrocarbons. n-Octane has been shown to produce a hydroperoxide at the terminal methyl carbon (Pope, Dykstra and Edgar, 1929). Autoxidation of n-decane

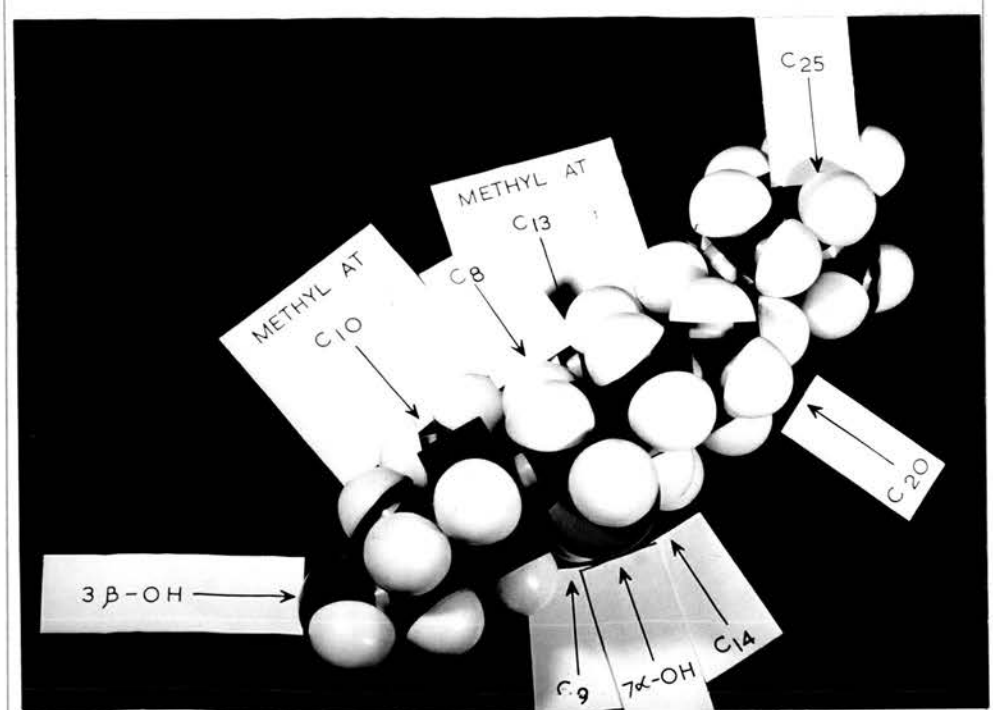
gave equimolar amounts of 2, 3, 4 and 5-hydroperoxides with a trace of 1-hydroperoxide (Benton and Wirth, 1953; Pritzkow and Muller, 1955). cis-Decalin produces the 9-hydroperoxide (Criegee, 1944) which can be easily made by autoxidation of cis-decalin at higher temperatures (170°) and reduced pressure. Oleates of cobalt, manganese, zinc and cerium have been shown to catalyse the reaction. Trans-decalin forms three different hydroperoxides (Kleinfeller, 1950). There are many other examples to show that hydroperoxides can be formed at saturated tertiary, secondary and even primary carbon atoms of the hydrocarbons (for other references see Hawkins, 1961).

On the basis of these findings it seems that almost any carbon atom in the cholesterol molecule is potentially capable of forming a hydroperoxide by autoxidation. In fact a number of hydroxylated derivatives of cholesterol have been isolated as presumably products of autoxidation (25- and 7α-hydroxycholesterols) (p. 4) and as cholesterol 'companions' (24, 22 and 20-hydroxy-

cholesterols) (p. 3). The different bile acids and their biosynthetic intermediates, the steroid hormones and their intermediates, constitute a large number of cholesterol derivatives, hydroxylated at different positions of the molecule. In the degradation of cholesterol side-chain to form bile acids and steroid hormones, 26-hydroxy (Danielsson, 1961e), 20-hydroxy (Shimizu, Hayano, Gut and Dorfman, 1961; Constantopoulos and Tchen, 1961) and 20, 22ϵ-dihydroxy (Constantopoulos, Satoh and Tchen, 1962; Shimizu, Gut and Dorfman, 1962) derivatives of cholesterol have been shown as intermediates. This shows that almost all the carbon atoms of the cholesterol side-chain can be hydroxylated, though in different tissues. Similarly most of the carbon atoms on the nucleus are also capable of hydroxylating, the important centres known being C_7, C_{11}, C_{12}, C_{16}, C_{17} and sometimes the angular methyl at C_{13}.

Since, for most of these hydroxylations it has been shown that the oxygen used is in a molecular state, it would seem that the mechanism of these hydroxylations is somewhat similar to that of the autoxidation reactions.

Thus, by analogy with the known hydroperoxidation reactions described above (autoxidations - photo-sensitized and catalysed) it seems that a hydroperoxidation at any of the carbon atoms in cholesterol, can be initiated by autoxidation with molecular oxygen. The yield of the hydrocarbon-hydroperoxides produced by autoxidations is low, nevertheless the reaction does occur and in biological systems the same reactions could be catalysed by the enzymes to give better conversions. These hydroperoxides could then be reduced to the respective hydroxylated derivatives by reduction with a reducing enzyme such as TPNH which has indeed been shown to be required for a number of biological hydroxylations. Although most of the carbon atoms in cholesterol could form hydroperoxides, some of them seem to be very difficult to attack because of stereo-electronic factors. Since the tertiary carbons are known to be more reactive than the secondary or primary, towards oxidation by different means,



MOLECULAR MODEL 7α-HYDROXY CHOLESTEROL

Figure 9

including autoxidation in air, it seems that the tertiary carbon atoms 25, 20, 17, 14, 9 and 8 would probably be the most susceptible to an attack by molecular oxygen. Of all the tertiary carbon atoms in the cholesterol molecule, the C_8 (β-hydrogen) is sterically hindered because of the two angular methyls (C_{10} and C_{13}) in the vicinity (see Figures 1 and 9). Carbon atoms 9 and 14 (α-hydrogens) are near the very reactive centre at C_7. A hydroxylation at that position (C_7) would be much easier than at any of these positions and once the position 7 has been attacked to form a hydroperoxide or a hydroxyl, C_9 and C_{14} are difficult to approach. Thus, apart from C_7, the most likely centres for an oxygen attack are the carbons 25, 20 and 17. The carbons 20 and 17 have indeed been shown to be important in the study of steroid hormones but the C_{25} has not been studied so well. A hydroxylation at C_{25} could be one of the stages in the cleavage of the cholesterol side-chain in the formation of bile acids but its importance can not be emphasized further because of the lack of information about this reaction from biological systems.

Although the hypothesis that the steroid hydroxylations may occur through hydroperoxide intermediates sounds quite reasonable, it has little experimental evidence to prove it. It would be of interest to test different relevant hydroperoxides in biological systems and see if they are converted into the hydroxylated products and/or the end products.

In this introduction an attempt has been made to show that in the degradation of cholesterol to bile acids there may be a large number of intermediates involved. The whole sequence of reactions involved in this degradation is, however, not yet known. A number of possible intermediates have been synthesized and used for *in vivo* and *in vitro* studies but still there are a large number of compounds which could be postulated as intermediates but have not yet been synthesized - e.g.

i) The mechanism of the initial hydroxylation at C_7 is not known and the possible intermediates have not been synthesized.

ii) The existing methods of synthesis of the intermediates involved in the epimerization of the hydroxyl at C_3 and saturation of the double bond are not very effective (see p. 89).

iii) A number of other possible intermediates have not been synthesized.

In this work an attempt has, therefore, been made i) to synthesize the possible intermediates involved in the 7α-hydroxylations;

ii) to devise improved methods of syntheses of the intermediates involved in epimerization at C_3 and saturation of the double bond;

iii) to devise methods of syntheses of other possible intermediates.

SECTION II

GENERAL PROCEDURES

ADSORPTION CHROMATOGRAPHY

Alumina and silicic acid have been used for the column chromatography of most products. A number of sterols, especially those carrying a 7α-hydroxyl function in the quasi-axial position, have been found to give dehydration products if put on active alumina. Several adsorption grades of alumina and silicic acid were thus tried, the preparations of which are described below.

'Active' Alumina

Alumina (activated, type 'O') obtained from Peter Spence was heated at 800° overnight and then allowed to cool slowly in the furnace to about 150°. It was then taken out and allowed to cool in a metal desiccator and stored in tightly stoppered bottles.

'Neutral' Alumina

Five hundred grammes of 'active' alumina were slurried with methanol and acidified with 3N-HCl to

about pH 3. The methanol was poured off and the alumina washed with water until the washings were neutral. It was then dried at 100° for 24 hours and stored.

'Deactivated' Alumina

The 'neutral' alumina was deactivated by adding a certain amount of water to it; a few ml. of water were added dropwise to the neutral alumina and mixed thoroughly. The proportions of water added varied for different preparations giving different grades of deactivated alumina, described at appropriate places in the text.

Silicic Acid

Two grades of silicic acid were used: ordinary silicic acid (100 mesh powder, obtained from Mallinckrodt Chemical Works, New York), and 'deactivated' silicic acid.

'Deactivated' Silicic Acid

Certain compounds which are unstable on alumina or silicic acid columns were found to be quite stable on thin-layer chromatograms. This fact led to the preparation of deactivated silicic acid which has almost the same activity as the silica gel on thin-layer. Silicic acid was washed with water several times and was then dried at 100° overnight to give 'deactivated' silicic acid.

THIN-LAYER CHROMATOGRAPHY

In recent years thin-layer chromatography (Kirschner et al. 1951, 1952; Stahl, 1953) has become a very useful tool in the separation of small amounts of materials (for review see Demole, 1961). It has been widely used for separation of small amounts of chemical products of biological reactions, but its use in organic chemistry has been rather limited. In the study of steroids, this method of chromatography has been very successful and is being widely used because of its superiority over the tedious method of paper chromatography. Thin-layer chromatograms are easy and quick to prepare and take only about an hour to develop and so thin-layer chromatography (TLC) can be used in a number of ways in the study of organic compounds. This method of chromatography can be further elaborated by the use of fluorescent thin-layer chromatograms (Boyd and Hutton, 1963). This is a non-destructive method for ultraviolet light absorbing materials which quench the fluorescence of the chromato-

gram under u.v. light source. The dark spots due to the u.v. absorbing material can be eluted directly from the plate.

A typical procedure used for this type of chromatography used in this work is outlined below.

The thin-layer chromatogram is prepared, as usual, by spreading over a glass plate a slurry of a mixture of 5% zinc silicate in Kieselgel (Kieselgel G. nach Stahl, Merck) in water. The plate is dried in the oven (100°) for 30 min. and then allowed to cool. The substance to be chromatographed is spotted on to the plate and the chromatogram developed in a suitable solvent system by ascending chromatography (about 1 hr. was typical for developing an 8 inch chromatogram). The plate is left at room temperature to evaporate the solvent and is then viewed under an ultraviolet light source and the u.v. absorbing materials, if any, are marked and, if required, eluted for further investigations.

This method of chromatography has been used in a number of different ways for the study of a number of organic reactions described here.

i) If the reaction is slow the reaction mixture can be sampled out at suitable intervals and chromatographed by TLC. The product or products can be found out at each time and the reaction can be stopped when the whole of the starting material disappears or when it seems to have reached an equilibrium. A number of specific colouring reagents have been used as sprays to develop coloured spots on the chromatogram. This was the usual method used for following most of the reactions described in this work. The details of different solvent systems and the colouring agents used are given at appropriate places.

ii) The purity of the compounds can be checked by running thin-layer chromatograms in different solvent systems; if the compound gives a single spot in all the systems it is quite a good indication of its purity. The same method can be applied for co-chromatography of a known compound with an unknown. In this work TLC has been used as one of the standard criteria of purity.

iii) This method was also used for checking different eluates obtained from adsorption chromatography on columns; each cut is concentrated and run on

a thin-layer chromatogram and the ones having similar spot or spots are pooled together. To check further if one spot is due to one component, the pooled eluates can be run in different solvent systems.

iv) Besides being used for following the reactions and checking purity of the compounds, this method also gives some further information. During the follow-up of a reaction mixture it can give information about the number and some properties of the side-products; their mobility on a certain solvent system can give an idea about the polarity of the molecule, the formation of certain products can be shown by using specific sprays.

The deficiencies of this method are that:

i) It is not quantitative.

ii) If there are a number of products formed it gives little information about the mode of formation of these products, whether they are a result of breakdown of the desired product or are formed directly from the starting material or are intermediates in the synthesis of the desired product.

iii) The compounds which do not give a simple colour reaction cannot be detected.

These difficulties can be overcome and this method can easily be made quantitative by using labelled substrates and coupling the TLC with radioactive counting; the reaction mixture is applied on to the chromatogram and it is then developed and cut into sections and eluted. In actual experiments, two spots of the reaction mixture were put on to a plate, the developed chromatogram was viewed under a u.v. light source and the dark spots, if any, due to a u.v. absorbing material were noted. One half of the chromatogram was then sprayed with a suitable colouring agent and the other half cut into eight parts (these cuts corresponded to spots and blank spaces of the chromatogram) (Fig. 25 and 27) and eluted with suitable solvents (chloroform, chloroform-ethanol or ether-ethanol). The solvents were evaporated and the residue counted by liquid scintillation counting.

Besides being quantitative this method can give a significant amount of other information about the reaction. The advantages of this method are:

i) Very small amounts of the substrates can be used and so it can even be used for fast reactions; a pilot experiment can be done on a very small amount and the time required for the reaction, the nature of products formed, etc., can be obtained by this method and then the reaction can be repeated with a larger amount.

ii) Reaction rates can be found. If a sample of the reaction mixture is chromatographed on TLC, the total radioactivity applied is given by the total counts from the plate and the proportion of different products at a particular time can be calculated corresponding to their respective radioactive counts.

iii) Whether the other products, besides the desired one, are a result of the breakdown of the desired product or are intermediates or side products can be found out by the decrease in the radioactive peak corresponding to the required product and increase in the radioactivity of other products or vice versa.

iv) The purity of labelled compounds can be checked by running the chromatograms in different

solvent systems and counting; it should have all the radioactivity accumulated in the cut corresponding to the substance.

v) If the product of the reaction contains a u.v. absorbing substance and a non-u.v. absorbing one running with the same mobility in different solvent systems, their relative amounts can be found out. The u.v. absorbing spot can be eluted from the plate and its optical density and thus its amount can be calculated, the same eluate can be evaporated and counted. The total count gives the count due to both constituents and the count for individual compounds can thus be calculated. An example of this could be in vitro studies using labelled cholesterol as substrate. If there is 26-hydroxycholesterol formed it will be difficult to find since it runs with the same mobility as 7-ketocholesterol on different solvent systems. By this method this problem can be resolved.

vi) The method can be used for kinetic studies of steroid reactions. Since the main purpose of studies undertaken in this work was the synthesis of possible intermediates, kinetic interpretations to the results have not been discussed.

vii) The method can also be applied to compounds which do not give a colour reaction on the plate.

For slow as well as fast reactions this method of TLC and counting can serve as a quantitative 'minute-to-minute' account of the reaction. A number of reactions have been studied by this method and the results are outlined later. The main purpose of these reaction studies was to find the time course for certain reactions and so the results have not been given other interpretations.

Radioactive Measurements

All radioactive measurements have been done by liquid scintillation counting on Packard Tricarb liquid scintillation counting system (model 314EX). The scintillator used was 5 g. PPO (2, 5-diphenyloxazole, scintillation grade; fluorescence max. 3800Å) and 0.3 g. dimethyl POPOP (1, 4-bis-2-(4-methyl-5-phenyl-oxazolyl)-benzene, scintillation grade; fluorescence max. 4300Å) in one litre of toluene; 10 ml. of this scintillator solution were used for each count. When

the eluates from TLC were to be counted, the eluate was taken into a counting vial and then evaporated; 10 ml. of the scintillator solution were added and the solution counted.

Ultraviolet Spectroscopy

The u.v. absorption measurements were done on spectrophotometer SP500 and the Optica recording spectrophotometer. The substance was usually read in an ethanol solution. For viewing fluorescent thin-layer chromatograms a u.v. lamp (mercury lamp; maximum emission 254 mμ) was used.

Infrared Spectroscopy

The infrared absorption measurements were carried out on an Infracord spectrophotometer (Perkin-Elmer) using the KBr disc method. The discs were prepared from 2 mg. samples of the compounds in about 200 mg. of KBr (dry, anhydrous); the compound was mixed with KBr under an infrared light

source and the disc prepared by pressing the material in a metal dye. A blank KBr disc was always used in the reference beam.

All the infrared spectrograms are given in the Appendix.

Radioactive Labelling

For the preparation of most of the labelled compounds, tritium was used as the labelling isotope. The labelling was done by the Wilzbach method (Wilzbach, 1957). It was found that longer exposures of the steroid with tritium led to several artifacts. The compounds were thus left in contact with tritium for a shorter time. For most of the labelled compounds 72 hr. were found sufficient to give a compound of reasonably high specific activity. By using the method of shorter exposure time the number of artifacts was reduced considerably and the purification of the products was found much easier and the yields were better. After 72 hr. the pressure was released and the unused tritium diluted with compressed air and allowed to escape through a fume cupboard. The

product was dissolved in a hydroxylic solvent like methanol and the solvent evaporated. The process was repeated several times to remove the 'labile' tritium. The labelling of certain compounds and further purification of the products is described later.

All radioactive compounds were stored in benzene at the deepfreeze temperature.

Melting Points and Optical Rotations

All melting points have been determined on a Kofler micro-stage (microscope RCH) and the optical rotations on Hilger polarimeter M412. All the $[\alpha]_D$ values given are for an approximately 2% solution of the compound in chloroform.

PHOTO-OXYGENATIONS

The photosensitized oxygenation reactions are well known but their use in syntheses has been rather limited. In these reactions the choice of solvent, the concentration of the substrate, the selection of a sensitizing dye and the form of the light source seem to be of a considerable importance.

Solvents

The photosensitized oxygenations have been conducted in different solvents (pyridine, benzene, alcohols, chloroform, etc.), the most widely used being pyridine and chloroform. If the oxygenations of steroids with a 5, 6-double bond are conducted in chloroform solution, the hydroperoxide group is introduced stereospecifically at the allylic 7α-position (Schenck, Gollnick and Neumuller, 1958b) without shifting the double bond, while with most other solvents the hydroperoxidation occurs at unsaturated tertiary carbon atom at position 5 with a subsequent allylic shift of the double bond into the 6, 7-position. The 5α-

hydroperoxide, cholest-5-en-3β-ol-5α-hydroperoxide (Schenck et al. 1957), is stable as a crystalline solid and also in pyridine solution but when left in suspension in chloroform or dioxan it undergoes rearrangement to form cholest-5-en-3β-ol-7α-hydroperoxide (Schenck, Gollinck and Neumuller, 1958 a; Lythgoe and Trippet, 1959).

The choice of the solvent, thus, depends on the desired mode of attack on the substrate (5α or 7α) and also on the solubility of the substrate and the photo-sensitizing agent. Since for this work 7α-peroxidation was the primary aim, most of the photo-oxygenations were conducted in chloroform solution.

The concentration of the substrate in the solvent is important since in dilute solutions in presence of a sensitizing agent the mode of oxygen attack is more specific and there are fewer breakdown products so the solutions used were quite dilute. In most of the reactions described later there was no trace of an ultraviolet absorbing material showing absence of any 7-keto compounds. Nevertheless, there were always some 7α-hydroxylated compounds formed by the break-down of the hydroperoxide.

All the substrates used in the described photo-oxygenations are very soluble in chloroform but most of the dyes used as sensitizers in previous photo-oxygenations are not very soluble in chloroform. A new catalyst, haematoporphyrin methyl ester, was thus developed for these reactions.

Catalysts (Sensitizing Dyes)

A large number of synthetic and natural dyes have been used as sensitizing agents for the photo-oxygenation reactions (Schenck et al. 1958; Oster et al. 1959; Livingstone and Owens, 1956). It has been shown that only dyes capable of photo-reduction can act as sensitizers and that the quantum yield of the reaction decreases markedly with the increasing dye concentrations (Oster et al. 1959). There are some dyes which act within 5 min. of the start of the oxygenation while some others take about 20 min.

Since for most of the photo-oxygenations described here, chloroform was the solvent (for reasons discussed elsewhere) only such dyes were chosen which are very

soluble in chloroform. For oxygenations where pyridine was used as the solvent haematoporphyrin, rose bengal, eosin, and protoporphyrin were used as photo-sensitizers but when the reaction was conducted in chloroform, haematoporphyrin methyl ester was used.

This new catalyst has been prepared from haematoporphyrin by different methylation procedures; methanolic HCl, dimethoxypropane and boron trifluoride-methanol complex have been used as methylating agents.

Light Source

Different light sources were tried. Fluorescent lamps (Phillips TL AD, 15 w, 18" long), Tungsten filament lamps 8" long), Rubidium lamp (8") and a HgZnCd lamp (Phillips, Holland; type 93145E), all gave the hydroperoxide but the fluorescent lamps were found to be the most satisfactory. With this lamp the temperature of the reaction mixture does not go above 20° C, while with

Tungsten filament lamps and the mercury-zinc-cadmium lamp, the temperature was high and so there were more side reactions. In all the experiments described two or four 15 watt fluorescent lamps were used.

The mercury-zinc-cadmium lamp was chosen because it has emission maxima in the same regions as the absorption maxima of haematoporphyrin methyl ester. But this lamp gave a high temperature, and so a number of breakdown products; though this lamp seems to be much more effective since all the starting material was used up within 5 hr. of the reaction while with other lamps it took a longer time. It seems possible to control the temperature by either water jacketing the reaction tube or passing dry cold air through the space between the light source and the reaction tube, but since the other lamps gave satisfactory results, no effort was made at controlling the temperature.

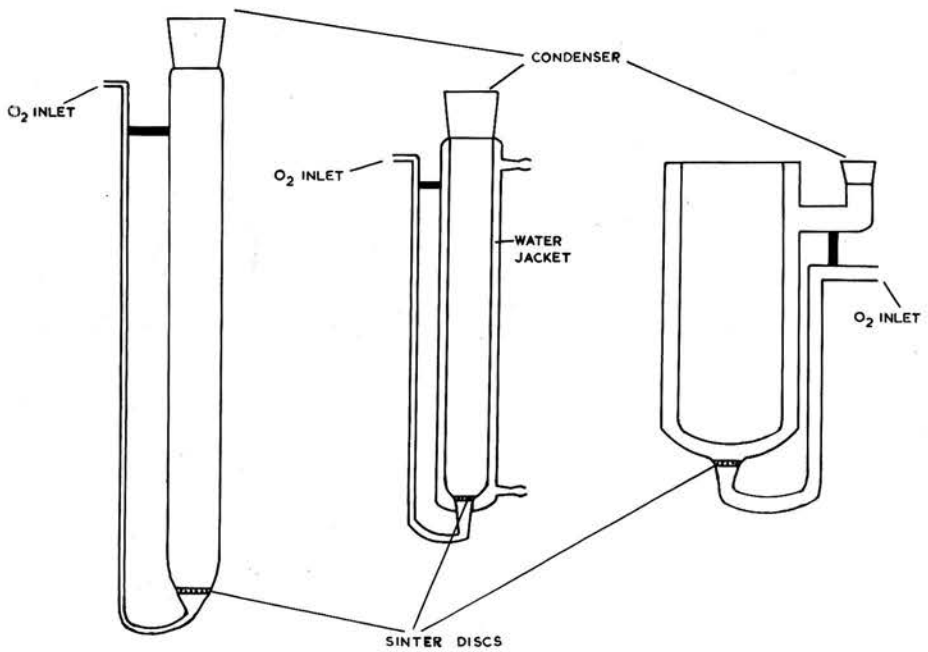


PHOTO-OXYGENATION REACTION TUBES

Figure 10

Reaction Tubes

Different reaction tubes were devised as to suit the volume of the solvent and the type of the light source used (Fig.10). With a single 70 w fluorescent lamp, Rubidium lamp and Hg. Zn. Cd. lamp the reaction tube was a tube-within-a-tube type of cylinder with the light source inside, while for other lamps a straight tube was used. In both types of reaction tube, the oxygen was admitted via a sintered disc at the bottom of the tube.

A typical method of photo-oxygenation and the following up of the reaction procedures is outlined below.

The steroid was dissolved in the solvent and the appropriate amount of the catalyst added. The solution was poured into the reaction tube (one of the types already described) and photo-oxygenated with a smooth stream of oxygen from a cylinder, the light source being one of the lamps described. (It was sometimes difficult to keep the volume of the solvent constant because of losses through evaporation. The volume was usually made up every hour by adding some fresh solvent.) The reaction mixture was tested every 2 hr. by taking out a sample and running on a thin-layer chromatogram. Two spots of the sample were put on a fluorescent thin-layer chromatoplate and run in a suitable solvent system. After drying and viewing under a u.v. lamp, one half of the plate was sprayed with potassium iodide (10% solution in ethanol-water 4:1 with a few drops of acetic acid); the hydroperoxide liberates iodine and a pale yellow colour develops

which deepens later. The other half of the plate was sprayed with phosphotungstic acid (PTA) which gives a bright pink colour to the hydroperoxides and different colours to the starting material and the breakdown products like the hydroxy compounds or the ketones.

The reaction was stopped after a suitable time and the product worked up as described under different preparations.

SECTION III

SYNTHESIS OF 7α-HYDROXYLATED COMPOUNDS

Introduction

As discussed earlier (p. 12) the 7α-hydroxylation seems to be one of the early stages in the degradation of cholesterol to bile acids and there is a possibility that this hydroxylation is brought about through hydroperoxide intermediates (p. 34 and p. 39). In such a case cholest-5-en-3β-ol-7α-hydroperoxide and cholest-5-en-3β, 7α-diol would be intermediates.

It has been shown that cholest-5-en-3β, 26-diol can be efficiently converted into chenodeoxycholic acid in the bile fistula rat, and in vitro it can hydroxylate at C_7 to give cholest-5-en-3β, 7α, 26-triol. If this 7α-hydroxylation also proceeds by the same mechanism, viz. the reduction of a hydroperoxide intermediate, then cholest-5-en-3β, 26-diol-7α-hydroperoxide would be an intermediate.

Photo-sensitized oxygenations have been used for the preparation of hydroperoxides. It has been shown (Schenck et al. 1958) that if the reactions are carried out in chloroform solution, the 5-6-unsaturated steroids

form the hydroperoxide in the allylic position 7; the oxygen attack is from the rear and thus results in the formation of 7α-hydroperoxides. This method of 7α-hydroxylation has great promise in the syntheses of 7α-hydroxylated derivatives of Δ^5-steroids. Since the oxygen attack in this method is very specific at the allylic 7α-position, a number of Δ^5-steroids having hydroxyl groups elsewhere in the molecule can be hydroxylated at C_7 without protecting the hydroxyls. A large number of cholesterol metabolites can thus be synthesized by this method.

In this work the photo-oxygenation reactions have been used to synthesize some 7α-hydroperoxy and 7α-hydroxy compounds which are possible intermediates in cholesterol degradation. Cholesterol and cholest-5-en-3β, 26-diol have been used to prepare the respective 7α-hydroperoxides and the 7α-hydroxy derivatives. The cholest-5-en-3β, 26-diol-7α-hydroperoxide was not isolated from the reaction mixture; the crude product was directly reduced by potassium iodide or sodium borohydride to give the cholest-5-en-3β, 7α, 26-triol which was isolated by chromatography.

It has been shown (Schenck et al. 1958) that if the photo-oxygenation is conducted in pyridine solution the product is a 5α-hydroperoxide which if left in suspension in chloroform rearranges to the 7α-hydroperoxide. Cholest-5-en-3β-ol-7α-hydroperoxide has been synthesized by two methods, viz. the isomerization of the 5α-hydroperoxide and by the direct photo-oxygenation of cholesterol in chloroform solution.

Preliminary experiments have been done on the photo-oxygenation of methyl 3β-hydroxy-5-cholenate in chloroform and the production of the hydroperoxide has been shown by liberation of iodine. The crude hydroperoxide was reduced with borohydride to give the corresponding hydroxy compound. The reduction product showed two spots corresponding, possibly, to the 7α- and 7β-hydroxylated derivatives and the separation of the two isomers could not be effected by chromatography or repeated crystallisation.

A quantitative study of the time course for the photo-oxygenation reaction using cholesterol as the model compound has been undertaken by using C^{14}-labelled cholesterol (Fig. 11 and 12). The photo-

oxygenation was carried out as described previously (p. 63), following the reaction by TLC.

Each chromatogram was cut into sections, eluted and counted. After 4 hours of the reaction about 22% of the total radioactivity was found in the cut corresponding to the hydroperoxide and the rest was in the cholesterol cut. The amount of the hydroperoxide increased during the first 16 hours and the amount of cholesterol decreased correspondingly. After 16 hours, however, a new product emerged having a mobility like that of the 7α-hydroxycholesterol and the relative amounts of the cholesterol and the hydroperoxide decreased. That this new product is probably the 7α-hydroxycholesterol is further indicated by its colour reaction with phosphotungstic acid (a blue colour) and a Lifschutz test. This product kept on increasing while the amounts of cholesterol and the hydroperoxide decreased over another 16 hours. This indicates that this product is being formed from the hydroperoxide and could very well be the 7α-hydroxycholesterol.

At 24 hours a new product R_F 0.72 giving a blue colour with phosphotungstic acid (PTA) appeared while the amounts of cholesterol, the hydroperoxide and the hydroxy compound all decreased. This seems to be a product formed from the 7α-hydroxycholesterol either by dehydration or some other reaction which removes or blocks one hydroxyl. The product is non-u.v. absorbing, has a mobility which suggests one nuclear hydroxyl and one other non-polar substituent (since its mobility is slightly less than cholesterol itself), it gives a blue colour with PTA. This product was not isolated for any further investigations.

From the above experiment it appears that under these conditions the production of the hydroperoxide goes steadily up during the first 16 hours and then it starts decomposing into other products. Thus 16 hours was taken as the standard time for this reaction. The oxygenation was kept on for 60 hours at the end of which there were a number of other u.v. absorbing and non-u.v. absorbing products formed. The whole plate was full of spots all having radioactivity (Fig. 12 and 11). It was not worked up to isolate any compounds.

Such experiments when repeated using four 15 w fluorescent lamps showed the same type of curves but the reaction was faster and the amount of the 7α-hydroperoxide reached its peak after 5 hours.

Cholest-5-en-3β, 7α-diol has been prepared by a photo-oxygenation of cholesterol and the subsequent reduction of the hydroperoxide. The existing method of synthesis of this compound by an allylic bromination (Henbest and Jones, 1948) does not give good yields and the 3β-hydroxyl has to be protected while in the photo-oxygenation method the hydroxyl need not be protected. This latter method can, thus, be used for 7α-hydroxylation of compounds having other hydroxyls in the molecule. Different methods have been used for the reduction of the hydroperoxide. The reduction with KI or with metal hydrides gave the same results. In every experiment there was, invariably, a trace of 7β-hydroxy compound formed which could be separated by crystallisation of the product. Since there was no trace of a u.v. absorbing material in the original photo-oxygenation product, the presence of the 7β-compound suggests that there is some 7β-hydroperoxide

formed in the photo-oxygenation reactions which gives the 7β-hydroxy product. That the compound is not the 5α-hydroxyl derivative, which could have been produced by the reduction of 5α-hydroperoxide in the photo-oxygenation product, is shown by co-chromatography of the product with an authentic sample of cholest-6-en-3β, 5α-diol; the 3β, 5α-diol has a different R_F from that of the 3β, 7β-diol. The product obtained here has the same R_F as the authentic cholest-5-en-3β, 7β-diol.

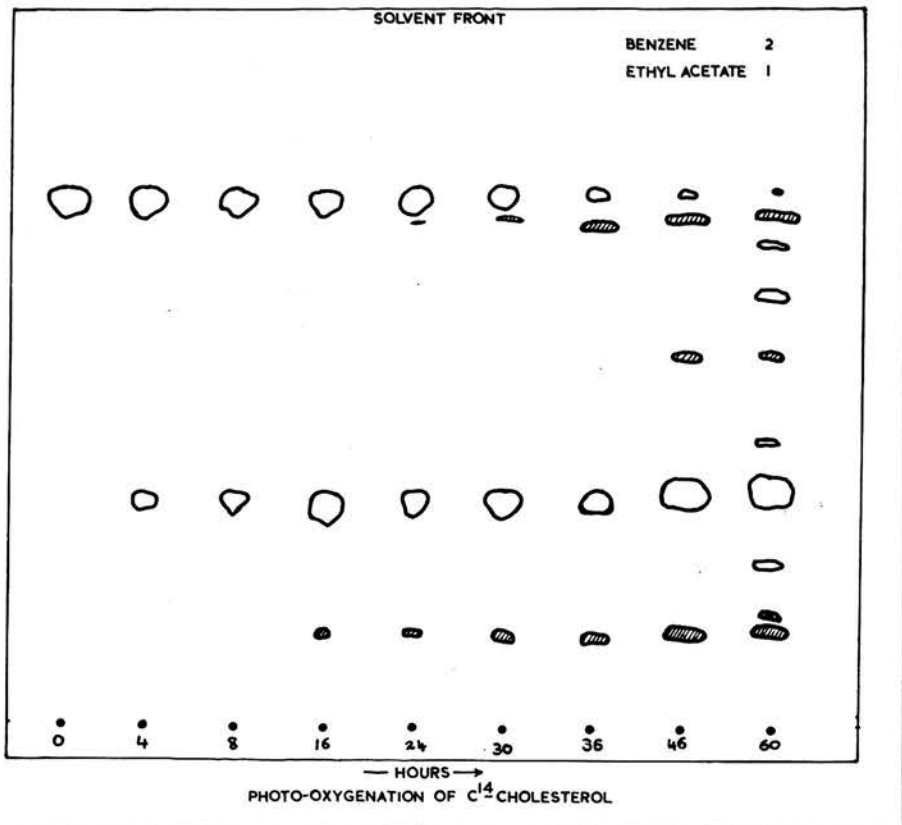


Figure 11

= **Blue with phosphotungstic acid**

= **Pink with phosphotungstic acid**

STUDY OF THE TIME COURSE OF THE PHOTO-OXYGENATION REACTION OF CHOLESTEROL

To study the reaction rates of these photo-oxygenations ^{14}C-labelled cholesterol was used and the reaction followed by thin-layer chromatography coupled with liquid scintillation counting (p. 63).

^{14}C-Labelled Cholesterol

. The ^{14}C-labelled cholesterol was obtained from rats given $^{14}C_2$-mevalonate. This sample of cholesterol was run on a thin-layer chromatoplate (benzene:ethyl acetate 2:1); two spots were put on one plate and after developing the chromatogram one half of the plate was sprayed with PTA which showed a spot corresponding to cholesterol and traces of some more polar material. The other half of the chromatogram was cut into eight sections and the sections eluted separately and counted. All activity was found in the cut corresponding to cholesterol. To remove the more polar material (though not radioactive) the crude product was chromatographed on active alumina (Peter Spence

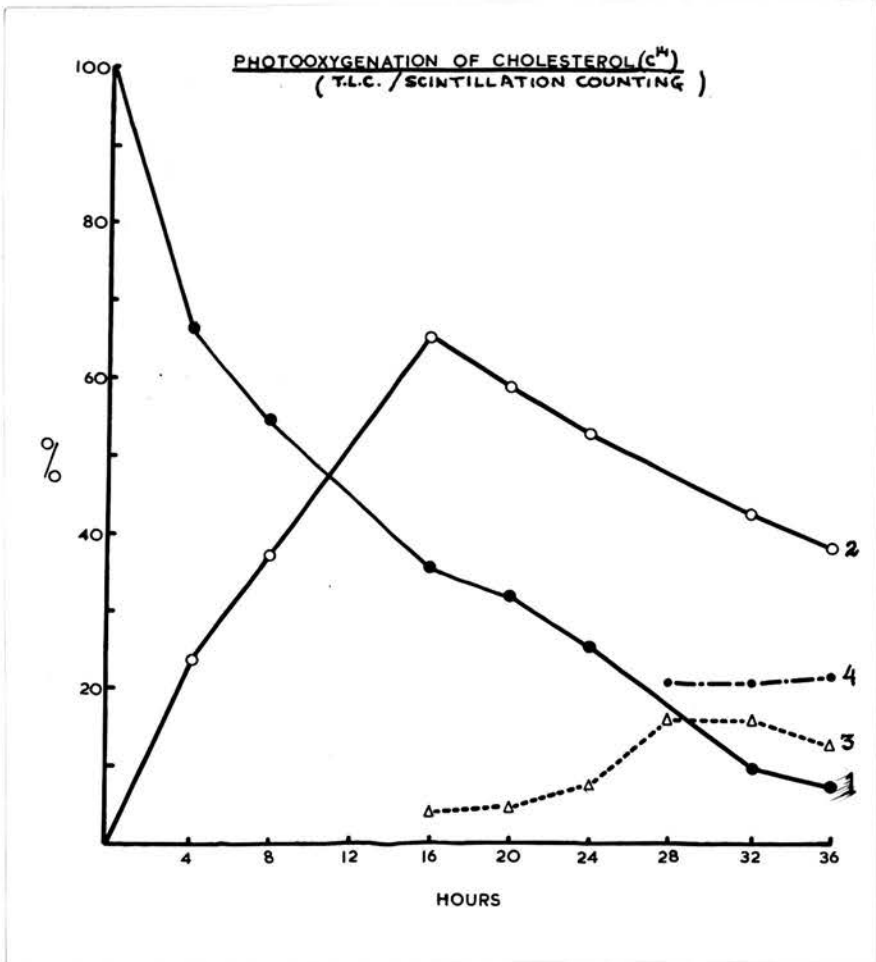


Figure 12

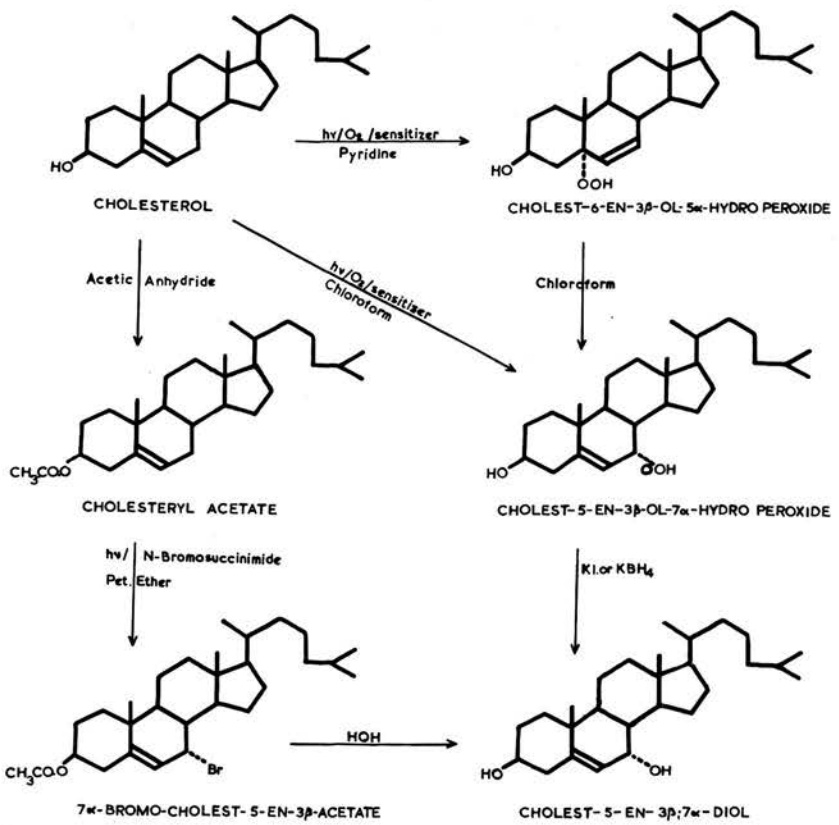
Count corresponding to:-

1. **Cholesterol.**
2. **7α-Hydroperoxide.**
3. **7α-Hydroxycholesterol.**
4. **Unknown.**

type 'O'). Elution with 50% and then 60% ether in benzene gave pure cholesterol (single spot on TLC and one radioactive peak); m.p. = 146°; specific activity = 22,225 c.p.m./mg.

Photo-Oxygenation of ^{14}C-Cholesterol

^{14}C-Labelled cholesterol (50 mg.) and 15 mg. haematoporphyrin methyl ester in 50 ml. chloroform were photo-oxygenated for 60 hours. A sample was taken out every 4 hours and two spots put on a fluorescent thin-layer chromatoplate. After developing the chromatogram (benzene:ethyl acetate 2:1) one half of the plate was sprayed with PTA while the other half cut into eight sections, eluted and counted as usual (pp. 50 & 63). The total count on the chromatogram and the count corresponding to different spots on the plate gave the respective percentages of the starting material and the different products. The results are summarized in the form of a graph (Fig. 12). At the end of the reaction the product was not worked up. The thin-layer chromatograms developed at different stages of the reaction are shown in Fig. 11.



METHODS OF SYNTHESIS OF CHOLEST-5-EN-3β,7α-DIOL.

Figure 13

Methods of Synthesis

Cholest-6-en-3β-ol-5α-hydroperoxide

Cholesterol (5.0 g.) and 40 mg. of haematoporphyrin were dissolved in 100 ml. of pyridine and photo-oxygenated for 20 hours. Evaporation of the solvent and crystallization of the residue from methanol left the photo-sensitizer dye in the supernatant. Two more crystallizations gave a colourless crystalline product.

m.p. = 141°	C & H	Theory	Found
$[\alpha]_D^{18}$ = +35		C = 77.51 %	77.29 %
IR (ν) = see Appendix Fig. [1]		H = 11.00 %	11.05 %

The experiment was repeated using eosin and also rose bengal as catalysts and it gave identical results.

Cholest-5-en-3β-ol-7α-hydroperoxide

1) By isomerization of cholest-6-en-3β-ol-5α-hydroperoxide

Cholest-6-en-3β-ol-5α-hydroperoxide (500 mg.) was left in suspension in pure dry chloroform obtained by filtration of the ordinary solvent through active

alumina. When ordinary chloroform was used the product showed several spots on TLC. After 12 hours the solution was evaporated to dryness under vacuum and the residue crystallized from benzene. Thin-layer chromatography (benzene:ethyl acetate 2:1) showed a trace of some more polar product which stained blue with phosphotungstic acid and had an R_F (0.2) like 7α-hydroxycholesterol. Repeated crystallization from benzene, methanol, petroleum ether-ethyl acetate or ether-ethyl acetate could not improve the product. The dry crystals were dissolved in 15% ether in petroleum ether (100 ml.) and chromatographed on 'deactivated' silicic acid (washed with water and dried at 100° overnight). Elution with 500 ml. of 30% ether in petroleum ether (60-80) gave the hydroperoxide shown by one spot on TLC and liberation of I_2. Evaporation of the eluates and crystallization of the solid product from benzene gave 350 mg. of pure cholest-5-en-3β-ol-7α-hydroperoxide.

	C & H	Theory	Found
m.p. = 147°		C = 77.45%	77.27%
$[\alpha]_D^{18}$ = -135		H = 11.08%	10.96%

IR (ν) = Appendix Fig. 2

ii) By direct photo-oxygenation of cholesterol

Cholesterol (4 g.) and haematoporphyrin methyl ester (40 mg.) were dissolved in chloroform (100 ml.) and photo-oxygenated (two 15 w fluorescent lamps); the reaction was followed by TLC as usual. After 20 hours the reaction was stopped and the solvent evaporated. The residue was dried and chromatographed on silicic acid as before. Elution with 20% and 25% ether in petroleum ether (60-80) gave the starting cholesterol and the hydroperoxide was eluted with 30% ether in petroleum ether. Evaporation of these eluates and crystallization of the residue from benzene gave pure cholest-5-en-3β-ol-7α-hydroperoxide. (The catalyst haematoporphyrin methyl ester was left on the column.)

m.p. = 149°

$[\alpha]_D^{18}$ = -135

IR (ν) = see Appendix Fig. # 2

Tritium labelled cholest-5-en-3β-ol-7α-hydroperoxide

Cholest-5-en-3β-ol-7α-hydroxperoxide (50 mg.) was left in contact with tritium in the Wilzbach apparatus (as described on p. 54) for 48 hours. The 'labile' tritium was removed in the usual way (p. 54) and a sample of the product checked on thin-layer; the thin-layer chromatogram was cut into sections, eluted and counted. The chromatogram showed two radioactive peaks, one major corresponding to the hydroperoxide and the other more polar. The product was chromatographed on 'deactivated' silicic acid (10 g.). The column was first washed with 15% ether in petroleum ether and the hydroperoxide eluted with 30% ether in petroleum ether (60-80) (200 ml.). Evaporation of the eluates and crystallization of the residue from benzene gave labelled cholest-5-en-3β-ol-7α-hydroperoxide shown by TLC and counting to be pure (one radioactive peak).

m.p. = 149°

Specific activity = 450,000 c.p.m./mg.

(counter efficiency = 40%)

Cholest-5-en-3β, 7α-diol

i) From cholest-5-en-3β-ol-7α-hydroperoxide

a) By reduction with potassium iodide

Cholest-5-en-3β-ol-7α-hydroperoxide (500 mg.) was dissolved in 60 ml. of ether-ethanol (1:5) and potassium iodide (2.5 g.) and acetic acid (3 drops) added and the salt dissolved by shaking. The mixture was then refluxed gently for 30 minutes. The solvent was evaporated, the product extracted with ether, washed with sodium thiosulphate (until colourless) and then with water. The dried ether extract was evaporated and the residue crystallized from methanol to give colourless crystals of cholest-5-en-3β, 7α-diol (400 mg.).

m.p. = 187°

$[\alpha]_D^{18}$ = -92

$C_{27}H_{46}O_2$ requires C = 80.59%
H = 11.4%
Found C = 80·1 %
H = 11·02 %

b) By reduction with lithium aluminium hydride

Cholest-5-en-3β-ol-7α-hydroperoxide (200 mg.) was dissolved in 5 ml. of ether and added dropwise to 250 mg. of lithium aluminium hydride. The tube containing the steroid was washed with another 5 ml. of

ether and added to the reaction flask and the mixture refluxed for 20 minutes. The excess of the reagent was destroyed with 20 ml. of ether-ethyl acetate (2:1) and the product poured into a saturated solution of sodium potassium tartarate and extracted with ether. The ether extracts were washed, dried and evaporated and the residue crystallized from methanol to give 120 mg. of crystals.

m.p. = 188°
Mixed m.p. = 188°

ii) From bromination method

The cholest-5-en-3β, 7α-diol obtained by the above two methods was compared with an authentic sample prepared by the allylic bromination method of Henbest and Jones (1948).

The modified method of allylic bromination and hydrolysis was as follows.

Cholesterol acetate (1.0 g.) was refluxed with 0.50 g. of N-bromosuccinimide in petroleum ether (60-66°) under light - the light and heat provided from two 150 w photoflood bulbs (2" away from the flask).

The product was filtered and the filtrate evaporated to dryness. Repeated crystallization from acetone gave the 7α-bromo-cholest-5-en-3β-acetate.

Cholest-5-en-7α-bromo-3β-ol-acetate (0.30 g.) was taken up in 2 ml. ether and treated with 0.30 g. of sodium formate in 3 ml. of formic acid at room temperature overnight. The product was extracted with ether, evaporated, and the residue hydrolysed by refluxing with sodium ethoxide in ethanol for 1 hour. The product was diluted with water, acidified, and extracted with ether. Evaporation of the dried extract and chromatography of the residue (230 mg.) on neutral alumina (10 g.) gave 7-hydroxycholesterol.

The eluates containing the diol (5% and 10% methanol in ether) showed a trace of some slightly less polar component R_F 0.42 (presumably 7β-hydroxy-cholesterol), in addition to the spot corresponding to 7α-hydroxycholesterol R_F 0.35 (TLC solvent system benzene:ethyl acetate:acetone, 10:5:3).

To remove this material the product was acetylated by refluxing in acetic anhydride and the diacetates chromatographed on neutral alumina (10 g.). Ten percent and then 20% benzene in petroleum ether eluted the less polar compound, and 30% benzene in petroleum ether and then benzene eluted the 7α-compound cholest-5-en-3β, 7α-diol-diacetate. Evaporation of these eluates and crystallization from methanol gave 3α, 7α-dihydroxycholesterol diacetate as colourless crystals.

The diacetate was hydrolysed with methanolic KOH at room temperature overnight. The product was extracted with ether, washed and dried. Evaporation of the extracts and crystallization of the residue from methanol gave colourless crystals.

m. p. = 187°

$[\alpha]_D^{18}$ = -92

IR (ν) = See Appendix Fig. 3

Cholest-5-en-3β, 7α, 26-triol

i) By photo-oxygenation of cholest-5-en-3β, 26-diol and the subsequent reduction of the 7α-hydroperoxide

Cholest-5-en-3β, 26-diol (250 mg.) was dissolved in chloroform (75 ml.) and 21 mg. of haematoporphyrin methyl ester added and the solution photo-oxygenated (four 15 w fluorescent lamps). The reaction was followed by TLC as usual. At the end of 6 hours the reaction was stopped and the solution treated with 1 g. of activated charcoal and filtered and the solid washed with 25 ml. of warm chloroform. The filtrate was evaporated under vacuum and the residue, crude cholest-5-en-3β, 26-diol-7α-hydroperoxide, reduced with KI or $LiAlH_4$.

Reduction with KI

The residue was reduced with 1.0 g. of KI in 25 ml. 1:5 ether-alcohol and the product worked up as usual. The residue obtained from the ether extracts showed several spots, on TLC, corresponding to the

starting diol and the two triols, cholest-5-en-3β, 7α, 26-triol and the isomeric 7β-compound. There were traces of several other less polar products. The residue was dried and chromatographed on alumina (Peter Spence, active type 'O'). Five percent ethanol in ether eluted the unchanged starting diol. The triols were eluted with 30% and then 50% ethanol in ether. The two eluates showed two spots, one bigger and a trace of the other, on TLC (R_F 0.2 and 0.15) (system - benzene:ethylacetate:acetone, 10:5:3) corresponding to the two isomeric triols. These eluates were evaporated and the residue crystallized from ethyl acetate. Several crystallizations from ethyl acetate gave a pure sample in the form of needles. Thin-layer chromatography showed only one spot.

m.p. = 232°

IR (ν) = See Appendix Fig. 4

In this photo-oxygenation although there were several other products produced, there was no trace of any u.v. absorbing material. TLC on fluorescent plates every 4 hours during the reaction did not show even a trace of u.v. absorbing material.

ii) Reduction with lithium aluminium hydride

In another run with 200 mg. of 26-hydroxy-cholesterol, the residue obtained after the removal of the catalyst was reduced with 150 mg. $LiAlH_4$ and the product worked up as usual. Chromatography and then repeated crystallization gave the cholest-5-en-3β, 7α, 26-triol.

m.p. = 232°

b) By photo-oxygenation of cholest-5-en-3β, 26-diacetate

Diacetate (200 mg.) and 15 mg. of haematoporphyrin methyl ester in 75 ml. of chloroform were photo-oxygenated as usual (solvent system for TLC, benzene:petroleum ether:ethyl acetate, 40:60:6). For the removal of the catalyst the reaction mixture was taken into a flask and petroleum ether (60-80) was added to make chloroform-petroleum ether (1:2). The solution was filtered through deactivated silicic acid and eluted with more chloroform-petroleum ether (1:2). The eluates were evaporated and the residue reduced with 200 mg. $LiAlH_4$ in 15 ml. of ether. The product was worked up as usual and crystallized from ethyl acetate. Repeated crystallization with ethyl acetate gave the triol which gave a single spot on TLC.

m.p. = 232°

IR (ν) = See Appendix Fig. 4

Attempted photo-oxygenation of 3β-hydroxy-chol-5-enate

Methyl-3β-hydroxy-5-cholenate (300 mg.) was dissolved in chloroform (100 ml.) and haematoporphyrin methyl ester (30 mg.) added and the mixture photo-oxygenated (four 15 watt fluorescent lamps). TLC (benzene:ethyl acetate, 2:1) at every hour showed the presence of a hydroperoxide (liberation of I_2 from KI) and a more polar material presumably the 7α-hydroxylated derivative, since it gave blue colour with phosphotungstic acid. The oxygenation was stopped after 6 hours, the catalyst was removed by treatment with charcoal and the clear solution evaporated, dried and the dried residue reduced with potassium iodide. The product showed several spots on thin-layer chromatography; the main spot gave a blue colour with phosphotungstic acid and had a mobility (on TLC) which suggested that it had two nuclear hydroxyl groups. This product could not be purified because of paucity of materials.

Summary

Thin-layer chromatography has been used for following a number of the steroid reactions described. The procedure of fluorescent TLC/scintillation counting developed in this work has been used in the study of photo-oxygenation reactions.

A method has been described which can be used for the synthesis of a number of 7α-hydroxylated Δ^5-steroids. Cholest-5-en-3β, 7α-diol has been synthesized through the 7α-hydroperoxy intermediate using this method of 7α-hydroxylation by photo-sensitized oxygenation.

It has been shown that it is possible to use this method for the 7α-hydroxylation of compounds having other hydroxyl or ester groups in the molecule. Cholest-5-en-3β, 7α, 26-triol has been synthesized and it has been shown that 3β, 7α-dihydroxy-chol-5-enic acid can also be prepared by this method.

Cholest-5-en-3β-ol-7α-hydroperoxide has been labelled with tritium.

SECTION IV

STUDIES ON POSSIBLE INTERMEDIATES INVOLVED IN THE INVERSION OF CONFIGURATION AT C_3 AND THE SATURATION OF THE DOUBLE BOND

Introduction

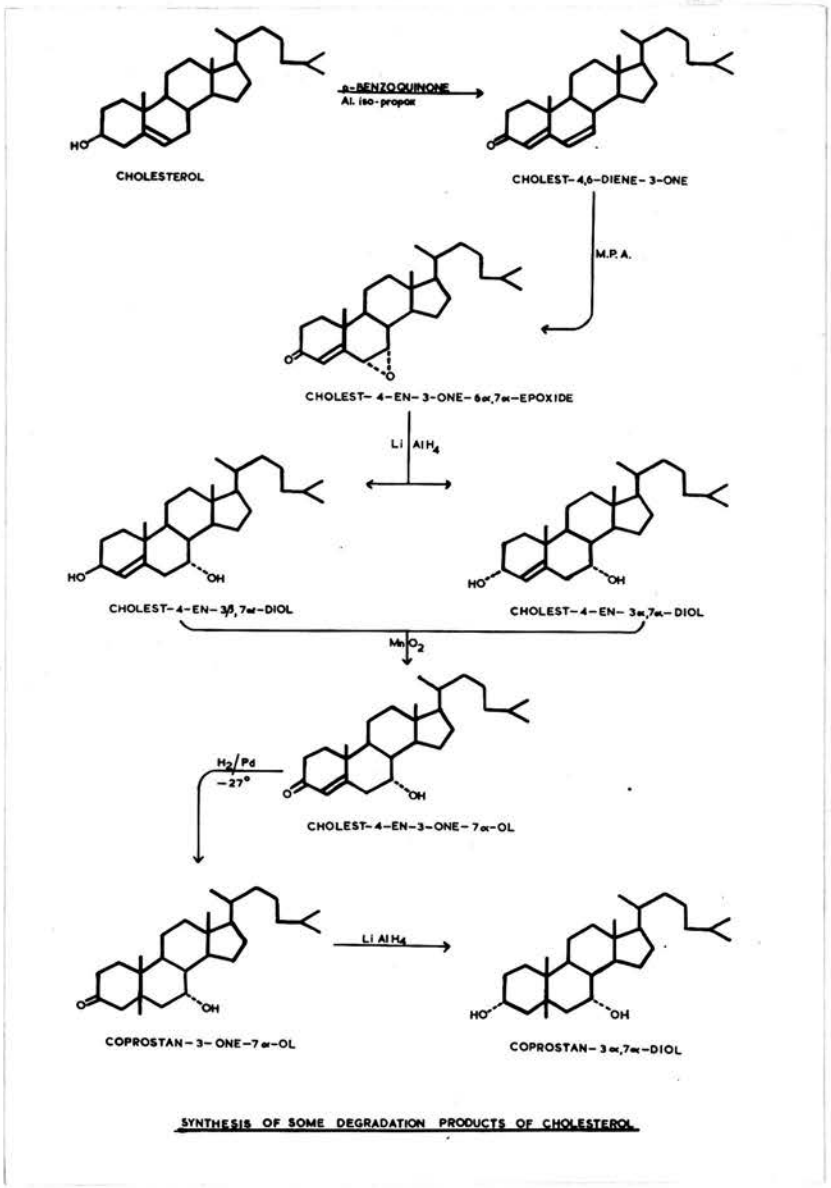
Cholest-4-en-3-one-7α-ol, coprostan-3-one-7α-ol and coprostan-3α, 7α-diol have been shown to be possible intermediates in the degradation of cholesterol to bile acids (p. 13). It has been shown that in the conversion of cholest-4-en-3-one-7α-ol to coprostan-3α, 7α-diol, cholest-4-en-3α, 7α-diol is another possible intermediate (p. 17).

Coprostan-3-one-7α-ol was first synthesized by Yamasaki et al. (1959a). While this work was in progress, Danielsson (1961b) published a new method of synthesis of this compound and a synthesis of cholest-4-en-3-one-7α-ol (Danielsson, 1961a). Cholest-4-en-3α, 7α-diol has not been described before while coprostan-3α, 7α-diol has been synthesized through a Kolbe's synthesis by electrolytic coupling of chenodeoxycholic acid with isovaleric acid (Bergstrom and Krabisch, 1957).

In the work described here all these compounds have been synthesized by new and easier methods.

The method of synthesis of cholest-4-en-3-one-7α-ol described by Danielsson involves many stages and the overall yield was very low. He claims 4-5% yield of the product cholest-4-en-3-one-7α-ol from 3β-benzoxy-7α-tetrahydro-2'-pyranyloxy-cholesterol. This latter compound has been prepared from 7α-hydroxycholesterol benzoate which was, in turn, prepared from cholesterol.

In the work described here, cholest-4-en-3-one-7α-ol has been prepared by a four stage synthesis from cholesterol giving an overall yield of about 13%. Cholesterol was oxidized by an Oppenauer oxidation to the cholest-4, 6-diene-3-one (Nickon and Bagli, 1961). This diene-one was treated with monoperphthalic acid in ether to get cholest-4-en-3-one-6α, 7α-epoxide (Nickon and Bagli, 1961). Attempts at selective cleavage of the epoxide ring to the 7α-hydroxy compound failed because of the vulnerability of the 3-keto group to such reagents. The epoxide was thus reduced with



SYNTHESIS OF SOME DEGRADATION PRODUCTS OF CHOLESTEROL

Figure 14

lithium aluminium hydride in tetrahydrofuran which gave a mixture of cholest-4-en-3α, 7α-diol and cholest-4-en-3β, 7α-diol. This mixture was re-oxidized selectively at the allylic position (C_3) using manganese dioxide (Sondheimer et al. 1953).

Oppenauer Oxidation of Cholesterol

Cholesterol was oxidized to cholest-4, 6-diene-3-one by an Oppenauer oxidation as used by Nickon and Bagli (1961). The separation of this compound from the reaction mixture was effected by chromatography on active alumina. Separation was not possible when ordinary alumina was used.

Epoxidation of Cholest-4, 6-diene-3-one

The cholest-4, 6-diene-3-one was treated with monoperphthalic acid in ether to get the epoxide, cholest-4-en-3-one-6α, 7α-epoxide. This epoxide was found to be very sensitive to active alumina; when a mixture of the diene-one and the epoxide, containing known amounts of both (calculated from the respective

u.v. absorption peaks at 284 mμ and 241 mμ) was chromatographed on active alumina, the amount of the diene-one isolated from the eluates was about 30% more than the amount originally put on. The amount of the epoxide had correspondingly decreased.

A new grade of deactivated alumina (p. 44) was then developed and the separation of the starting diene-one and the isolation of pure cholest-4-en-3-one-6α, 7α-epoxide effected by chromatography of the mixture on this alumina.

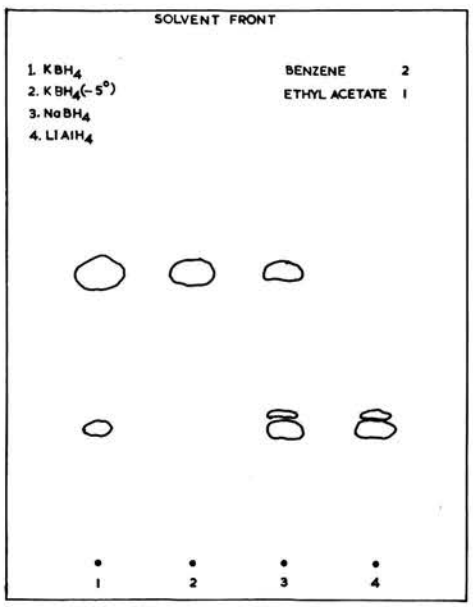
The epoxidation was carried out using monoperphthalic acid since perbenzoic acid did not give good results (cf. Nickon and Bagli, 1961). To establish the right conditions for this reaction the conditions were varied and it was found that the reaction went better in an excess of the per acid in dilute solution. These experiments were done on quite small amounts of the cholest-4, 6-diene-3-one and the amounts of the products calculated from ultraviolet spectrophotometric determinations; the optical density at 284 mμ and 241 mμ was measured and the respective amounts

calculated (cholest-4, 6-diene-3-one, $\epsilon_{284} = 29,000$; cholest-4-en-3-one-6α, 7α-epoxide, $\epsilon_{241} = 16,000$). No effort was made to isolate the compounds.

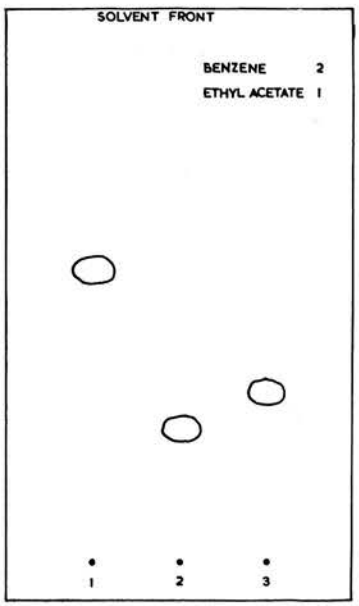
Care must be taken using an oxidizing agent as powerful as monoperphthalic acid and so it was essential to investigate the best conditions for the specific attack on the Δ^6-bond to the 6α, 7α-epoxide with the minimum side reactions.

Thus a procedure was developed to study the rates of this reaction at different temperatures. The procedure for these studies is outlined on p. 102 and the results given in the form of a graph (Fig. 18).

On the basis of these findings, future experiments were conducted with an excess of per acid in a dilute solution and at 37° C. The work-up procedure and the chromatography of the product is described later (p. 103).



A. METAL-HYDRIDE REDUCTION OF THE EPOXIDE



B. SOME PURIFIED COMPOUNDS

Figure 15

B.

1. **Cholest-4-en-3α-ol-6α, 7α-epoxide.**
2. **Cholest-4-en-3α, 7α-diol.**
3. **Coprostan-3α, 7α-diol.**

Reduction of the Epoxide

The reductive cleavage of epoxide rings gives trans-diaxial substituted products (Barton, 1953; see also Fieser and Fieser, 1959) and the reduction of steroid Δ^4-3-ketone gives a mixture of 3α- and 3β-hydroxylated compounds (McKennis and Gaffney, 1948; Plattner et al. 1948). Thus cholest-4-en-3-one-6α, 7α-epoxide would be expected to give a mixture of cholest-4-en-3α, 7α-diol and cholest-4-en-3β, 7α-diol if reduced with metal hydrides. However, potassium borohydride and sodium borohydride seem to reduce the 3-keto group in preference to the epoxide ring. Thin-layer chromatography of the product of reduction of the epoxide with these borohydride reagents showed several spots corresponding to the unchanged epoxide, the two expected diols and two products which were more polar than the epoxide and less polar than the diols. Their mobility (R_F = 0.53 in benzene:ethyl acetate (2:1) system) suggested (Fig. 15) the presence of one nuclear hydroxyl group along with a group like the

epoxide. At reduced temperatures the selectivity of these reducing reagents increases (Fig. 15) and the amount of the products corresponding to the diols decreases (Fig. 15). Potassium borohydride actually appeared to be more selective than sodium borohydride (Fig. 15).

The epoxide when reduced with potassium borohydride in methanol at -5° gave a product whose properties suggested it to be cholest-4-en-3α-ol-6α, 7α-epoxide. The procedure for the synthesis of this compound is described later (p. 106) and the proof for its structure discussed in Section VI.

In the reduction with these metal hydride reagents the 3α-isomer seems to be the predominant of the two isomers 3α-ol and 3β-ol. This selectivity seems to decrease from potassium borohydride to sodium borohydride and lithium aluminium hydride, the last being the least stereo-selective.

Since borohydride reagents gave a mixture of products and the reduction was not complete, the cholest-4-en-3-one-6α, 7α-epoxide was reduced with lithium aluminium hydride in tetrahydrofuran which gave as the product a mixture of cholest-4-en-3α, 7α-diol and cholest-4-en-3β, 7α-diol which was used for the next stage of the synthesis.

Since these reductions give the 3α-isomer as the predominant of the two products, it is much easier to remove the 3β-isomer either by repeated crystallization or more easily by treatment with digitonin. A little of the mixture of the diols was treated with digitonin and cholest-4-en-3α, 7α-diol isolated. This compound has not been described before.

Reoxidation of the Mixture of Diols

Manganese dioxide has been found to be a selective oxidizing agent for allylic hydroxyl groups (Sondheimer et al. 1953). The mixture of the diols obtained by the reduction of the epoxide was oxidized with manganese dioxide in chloroform. To study the effect of temperature on the reaction two experiments were set

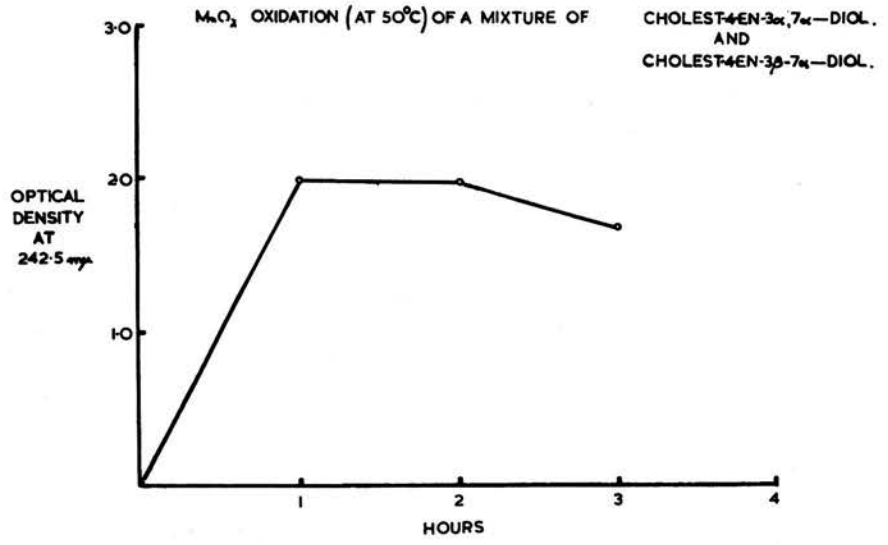

MnO_2 OXIDATION (AT 50°C) OF A MIXTURE OF
CHOLEST-4-EN-3α,7α—DIOL.
AND
CHOLEST-4-EN-3β-7α—DIOL.
OPTICAL DENSITY AT 242·5 mμ
3·0
2·0
1·0
1
2
3
4
HOURS

Figure 16

up, one at room temperature and the other at 50°. The one at 50° showed that the amount of the product started decreasing after 2 hours and after 3 hours it showed a trace of material absorbing at 284 mμ (Fig. 16) but when conducted at the room temperature it did not show any trace of material absorbing at 284 mμ even after 24 hours.

The reaction was repeated at room temperature using 580 mg. of the mixture of the diols. The product was sampled every hour and used for ultraviolet spectrophotometry and thin-layer chromatography; the optical density at 242.5 mμ was measured and the amount of total product formed calculated. The results are given in the form of a graph which shows that about 60% of the starting material was converted into the product cholest-4-en-3-one-7α-ol within the first hour of the reaction. That the reaction was complete after 8 hours was shown by TLC and ultraviolet spectrophotometric determinations. The product did not show any trace of material absorbing at 284 mμ even after 24 hours.

The compound, cholest-4-en-3-one-7α-ol, has been found to be unstable on alumina (Danielsson, 1961a), the 7-hydroxyl being in the less preferred axial (α) configuration undergoes dehydration to cholest-4, 6-diene-3-one. But when deactivated alumina was used for its chromatography, dehydration did not occur and the product was isolated pure.

The experimental procedures for this oxidation and the purification of the product are outlined later.

Selective Reduction of the 4, 5-Double Bond in Cholest-4-en-3-one-7α-ol

A selective reduction of the 4, 5-double bonds of steroids to a 5β-hydrogen and thus compounds of the coprostane series, can be brought about by hydrogenation at a low temperature (Grasshof, 1934; Nickon and Bagli, 1961). These findings were applied to cholest-4-en-3-one-7α-ol and the compound was hydrogenated at -27°. The reaction was studied by

following it every hour by the usual methods of TLC and spectrophotometry. Five hours' hydrogenation was found to be sufficient to reduce the double bond without affecting the keto group at position 3 (Fig. 21 and 22). By this method coprostan-3-one-7α-ol was obtained in good yield.

Reduction of the Coprostan-3-one-7α-ol

The reduction of 3-ketones of the coprostane series is known to give the 3α-isomer as the major product, when the reaction is conducted in a neutral medium (Ruzicka et al. 1934; Barton et al. 1949).

Coprostan-3-one-7α-ol was reduced with lithium aluminium hydride or sodium borohydride to give coprostan-3α, 7α-diol. The same product can also be obtained if cholest-4-en-3-one-7α-ol is hydrogenated at room temperature in a neutral medium.

The hydrogenation at room temperature is not selective and also affects the 3-ketone. In view of the fact that the reduction of the 3-ketone of the coprostane series predominantly gives the 3α-isomer it is not

surprising that in both cases the product was predominantly the 3α-isomer (shown by TLC). The 3β-isomer can easily be removed by crystallization of the product or by treatment with digitonin. That the product is coprostan-3α, 7α-diol has been shown by comparison with an authentic sample prepared by a Kolbe's synthesis as described by Bergstrom and Krabisch (1957).

The advantages of this route for the synthesis of coprostan-3α, 7α-diol and analogues of this substance are: the overall yields are good while the starting material, cholesterol, is inexpensive. The chemical synthesis proceeds through the same intermediates as proposed for biological degradations. The method can be applied to a large number of Δ^5-steroids having hydroxyl groups elsewhere in the molecule and so a number of possible intermediates can be synthesized by this procedure.

Methods of Synthesis

Cholest-4, 6-diene-3-one

The compound was obtained by Oppenauer oxidation of cholesterol according to Nickon and Bagli (1961).

Cholesterol (75 g.) and 450 g. of p-benzoquinone, freshly crystallized from petroleum ether (100-120) in 4.375 litres of pure dry toluene (previously purified with concentrated H_2SO_4, washed, dried over $CaCl_2$ and redistilled over sodium) was heated to reflux temperature and 100 g. of powdered aluminium iso-propoxide was added in small portions with sufficient time to allow the vigorous reaction to subside. The mixture was refluxed for 2 hours and then diluted very cautiously with 2.5 litres of hot water and steam-distilled for 5 hours. All the toluene distilled over in about 4 hours. The reaction mixture was brought to 0° and acidified with 1N-H_2SO_4. Constant stirring during the addition of the acid gave a fine slurry of the reaction mixture which was left at 3°C overnight

and then filtered under suction. The solid and the filtrate were extracted with ether separately. The ether extracts were pooled together, washed with 1N-H_2SO_4 and then with water, bicarbonate, and again with water. Evaporation of the dried ether extracts gave a dark brown residue which was thoroughly dried under vacuum and dissolved in dry benzene and chromatographed on 750 g. of 'activated' alumina (p. 43). Elutions with benzene, 10% ether in benzene and then 20% ether in benzene, took off all the cholest-4, 6-diene-3-one leaving a brown residue on the column. The different eluates were shown by thin-layer chromatography (benzene:petroleum ether:ethyl acetate, 40:20:5) to have only one spot (R_F 0.58 Evaporation of the combined eluates gave a pale yellow residue which on crystallization from methanol gave colourless needles (35 g.).

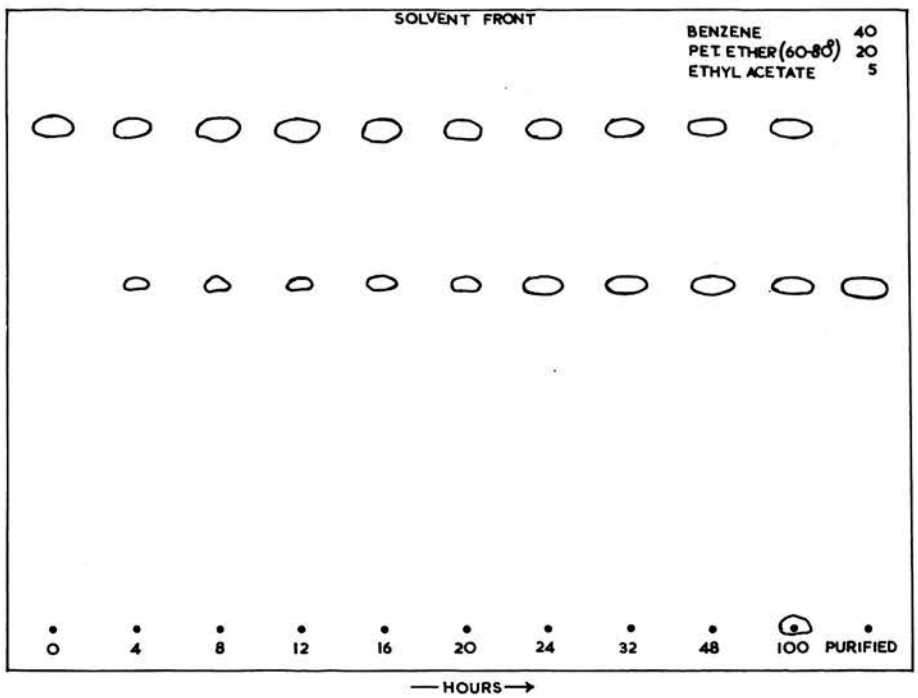
m.p. = 81.5°C C and H ($C_{27}H_{42}O$)

$\lambda_{max.}$ = 284 mμ Found C = 84.70% Theory C = 84.75%

ϵ_{284} = 29,000 H = 11.28% H = 11.07%

$[\alpha]_D^{17}$ = +36

IR (ν) = See Appendix Fig. 5



MONOPERPHTHALIC OXIDATION OF CHOLEST-4,6-DIENE-3-ONE

Figure 17

Study of the Reaction Rate of the Epoxidation Reaction

i) At room temperature (18° C)

Cholest-4, 6-diene-3-one (0.100 g.) was dissolved in 5 ml. of ether solution of monoperphthalic acid containing 0.155 g. of the acid, and 1.5 ml. of chloroform was added and the mixture left at room temperature. A sample was taken out every 4 hours and two spots put on a fluorescent thin-layer plate and the chromatogram developed in benzene:petroleum ether (60÷80°):ethyl acetate (40:20:5) solvent system. The chromatogram was viewed under a u.v. lamp and the two dark spots corresponding to cholest-4, 6-diene-3-one and cholest-4-en-3-one-6α, 7α-epoxide were eluted separately (ether alcohol) and the optical densities measured at 284 mμ and 241 mμ respectively. The other half was sprayed with phosphomolybdic acid (PMA) in ethanol solution (1%) to find if there were any other products formed.

The results obtained are plotted in the form of a graph (Fig. 18) which shows that within 5 hours the amount of the epoxide formed is about as much as the amount of unchanged cholest-4, 6-diene-3-one. The reaction then slows down and reaches an equilibrium in 70 hours.

At the end of 100 hours, the reaction mixture was filtered through 'neutral' alumina (10 g.) and eluted with another 50 ml. of chloroform. The eluates were evaporated and the residue (0.072 g.) was dissolved in petroleum ether (60-80) and chromatographed on 'deactivated' alumina (3% water in neutral alumina). The column was washed with petroleum ether and then 10% benzene in petroleum ether. Elution with 20% and 25% benzene in petroleum ether gave the starting diene-one. Thirty percent of benzene in petroleum ether started eluting the epoxide also. Continued elution with 30% benzene in petroleum ether and then benzene gave the epoxide; these eluates were evaporated and the residue crystallized from methanol to give fine colourless needles (0.031 g.).

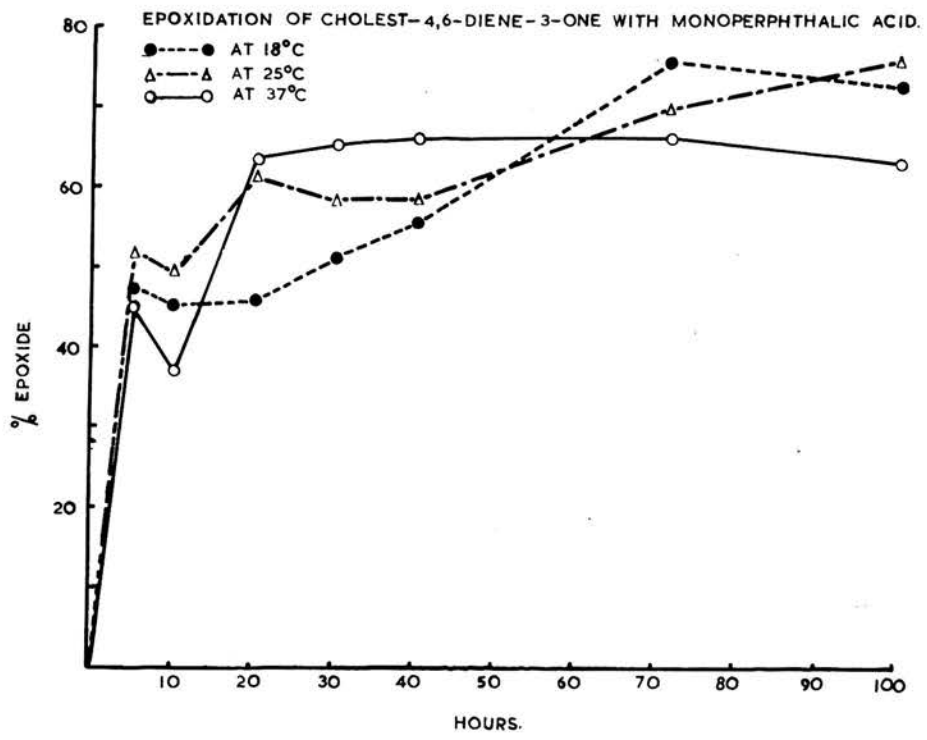

EPOXIDATION OF CHOLEST-4,6-DIENE-3-ONE WITH MONOPERPHTHALIC ACID.
AT 18°C
AT 25°C
AT 37°C
% EPOXIDE
80
60
40
20
10
20
30
40
50
60
70
80
90
100
HOURS.

Figure 18

m.p. = 136° $[\alpha]_D^{18}$ = -57

λ max. = 241 mμ IR (ν) = See Appendix Fig. 6

ϵ_{214} = 15,600

ii) At 25°

The reaction was repeated with the same amount of the starting material and followed in the same way and worked up as usual. The results are summarized in Fig. 18.

iii) At 37°

The results obtained by performing the reaction at 37° are summarized in Fig. 18.

Cholest-4-en-3-one-6α, 7α-epoxide

Cholest-4, 6-diene-3-one (5.2 g.) was dissolved in 80 ml. of ether solution of monoperphthalic acid (containing 8 g. of the acid) and 25 ml. of chloroform added and the mixture left at 37°. The reaction was stopped after 25 hours and worked up as described.

The residue (3.66 g.) was chromatographed on 'deactivated' alumina (25 g.) (3% water in neutral alumina) and the unchanged starting material eluted with 20% and then 25% benzene in petroleum ether. The epoxide was eluted with 30% benzene in petroleum ether. Evaporation of these eluates and crystallization of the residue from methanol gave colourless needles (1.76 g.).

m.p. = 136° C and H ($C_{27}H_{42}O_2$)

ϵ_{241} = 15,600 Theory C = 81.4% Found C = 81.3%

$[\alpha]_D^{18}$ = -57° H = 10.45 % H = 11.04%

IR (ν) = See Appendix Fig. 6

REDUCTION OF CHOLEST-4-EN-3-ONE-6α, 7α-EPOXIDE

i) With Potassium Borohydride at -5°

Cholest-4-en-3-one-6α, 7α-epoxide (100 mg.) was dissolved in methanol (10 ml.) and cooled to -5° and added to a cooled suspension of potassium borohydride (100 mg.) in methanol (5 ml.). The reaction mixture was kept stirred and was tested by fluorescent TLC after every 10 minutes; the samples showed two spots, one u.v. absorbing corresponding to the starting material and the other non-u.v. absorbing which gave a brownish colour with phosphotungstic acid. After 4 hours, analysis on a thin-layer chromatogram showed a very slight trace of some more polar material presumably the diol. The reaction was stopped after 5 hours; the excess of the reagent was decomposed with water and the product extracted with ether. Evaporation of the residue and crystallization from methanol gave 58 mg. of cholest-4-en-3α-ol-6α, 7α-epoxide shown as pure by TLC and other methods.

$C_{27}H_{44}O_2$ requires C = 81.0%

H = 11.0%

m.p. = 108° Found C = 81.65%

H = 11.15%

IR (ν) = See Appendix Fig. 7

This compound (15 mg.) when oxidized with manganese dioxide (150 mg.) in chloroform (5 ml.) for 4½ hours gave the starting epoxide shown by mixed melting point, ultraviolet and infrared spectroscopy and co-chromatography on TLC.

The compound (cholest-4-en-3α-ol-6α, 7α-epoxide) when reduced with lithium aluminium hydride gave a compound identical with cholest-4-en-3α, 7α-diol.

ii) <u>Reduction with Lithium Aluminium Hydride</u>

(Mixture of cholest-4-en-3α, 7α-diol and cholest-4-en-3β, 7α-diol)

Cholest-4-en-3-one-6α, 7α-epoxide (630 mg.) in 7 ml. of tetrahydrofuran (freshly distilled over sodium)

was added slowly to a suspension of lithium aluminium hydride (1.0 g.) in tetrahydrofuran (3 ml.) and then refluxed for 30 minutes. After cooling, the excess reagent was decomposed with ether-ethyl acetate and the whole poured into a saturated solution of Rochelle salt and extracted with ether. The ether solution was dried over sodium sulphate and evaporated to dryness giving 590 mg. of a residue containing cholest-4-en-3α, 7α-diol and cholest-4-en-3β, 7α-diol. The two isomers were not resolved at this stage and were used for the next preparation as such.

Thin-layer chromatography (benzene:ethyl acetate: acetone, 100:50:30) showed two spots corresponding to the 3β, 7α-diol and the 3α, 7α-diol. Infrared spectrum showed large hydroxyl peaks and no carbonyl. That the reduction was complete was also shown by the absence of ultraviolet absorbing material at 241 mμ.

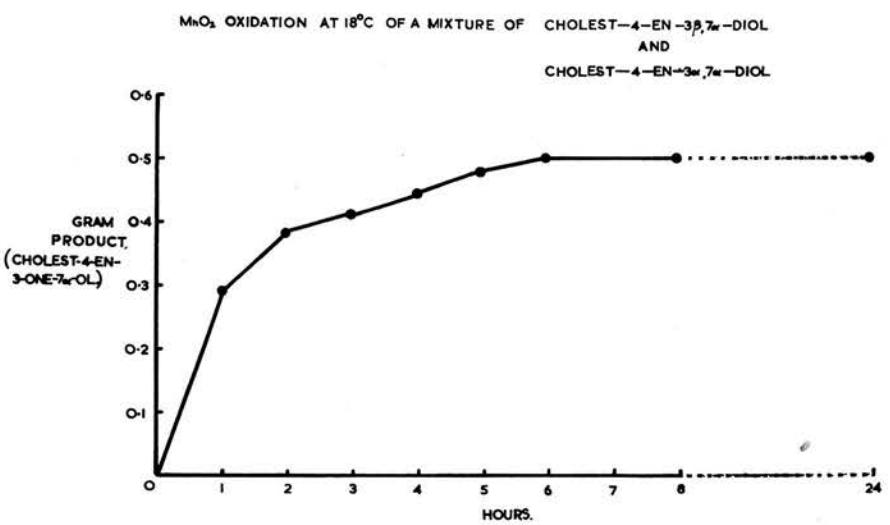
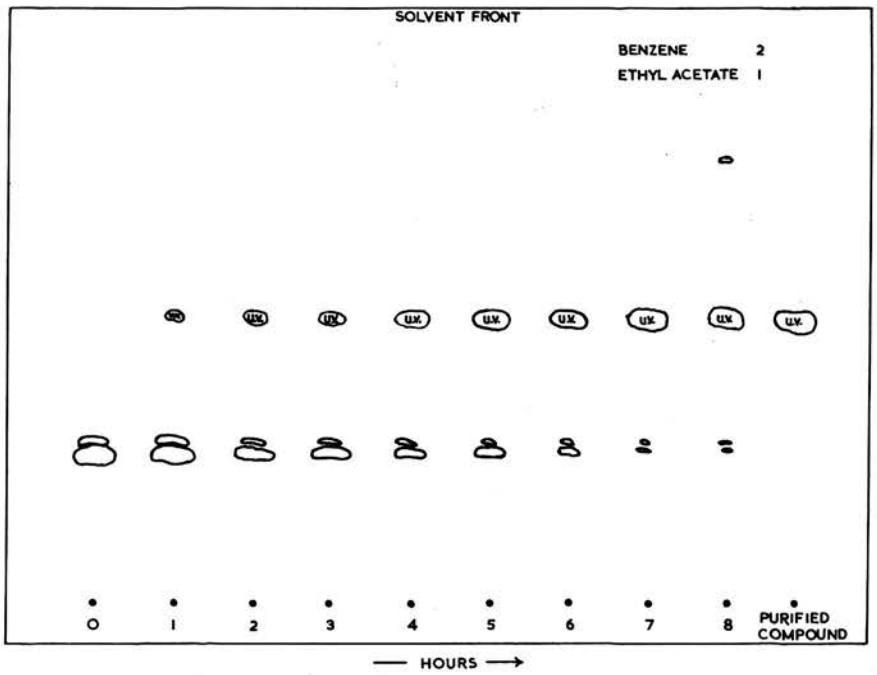

MnO2 OXIDATION AT 18°C OF A MIXTURE OF CHOLEST—4—EN—3β,7α—DIOL
AND
CHOLEST—4—EN—3α,7α—DIOL
GRAM PRODUCT, (CHOLEST-4-EN-3-ONE-7α-OL)
0·6
0·5
0·4
0·3
0·2
0·1
0
1
2
3
4
5
6
7
8
24
HOURS.

Figure 19

Cholest-4-en-3-one-7α-ol

The mixture of the diols cholest-4-en-3α, 7α-diol and cholest-4-en-3β, 7α-diol (580 mg.) was dissolved in chloroform (80 ml.) and manganese dioxide (5 g.) added. The mixture was shaken for 12 hours at room temperature, and 1 ml. aliquots were taken out every 2 hours and used for ultraviolet spectrophotometric determinations and thin-layer chromatography. After 12 hours the product was filtered and washed with hot chloroform, the filtrate evaporated and the residue dried thoroughly by suction. The dry residue (550 mg.) was dissolved in 90% benzene in petroleum ether and chromatographed on 'deactivated' alumina (40 g.) (10 ml. water per 100 g. of neutral alumina). The column was washed with benzene and cholest-4-en-3-one-7α-ol, eluted with 2% ether in benzene and then 5% ether in benzene. The combined eluates were evaporated to dryness in vacuo and crystallized from ethyl acetate giving 489 mg. of colourless needles.



MnO_2 OXIDATION OF THE MIXTURE OF CHOLEST-4-EN-3α,7α-DIOL & 3β,7α-DIOL.

Figure 20

C and H ($C_{27}H_{43}O_2$)

m.p. = 183.5-184.5° Found C = 80.92% Theory C = 80.95%

$\lambda_{max.}$ = 242.5 mμ H = 11.2% H = 11.1%

$\epsilon_{242.5}$ = 15,000 $[\alpha]_D^{17}$ = +67

IR (ν) = See Appendix Fig. 9

Further elution with 10% ether in benzene and then ether, eluted the unchanged 'diol mixture'. These eluates were pooled and added to the previous test aliquots and evaporated under vacuum. The dried residue was reoxidized with MnO_2 in $CHCl_3$. This time the mixture was left shaking for 24 hours. It did not show any absorption peak at 284 mμ and the same work-up procedure as before gave another 37 mg. of cholest-4-en-3-one-7α-ol.

Coprostan-3-one-7α-ol

By selective hydrogenation of cholest-4-en-3-one-7α-ol.

i) Study of the Rate of Reaction

Cholest-4-en-3-one-7α-ol (15 mg.) was dissolved in ether (5 ml.) and 10% palladium-on-charcoal (4 mg.)

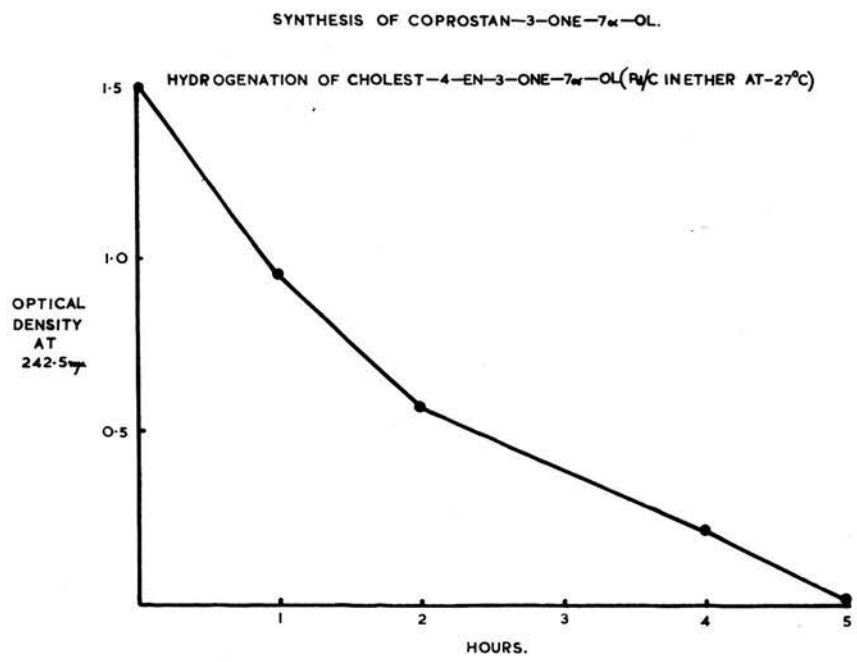
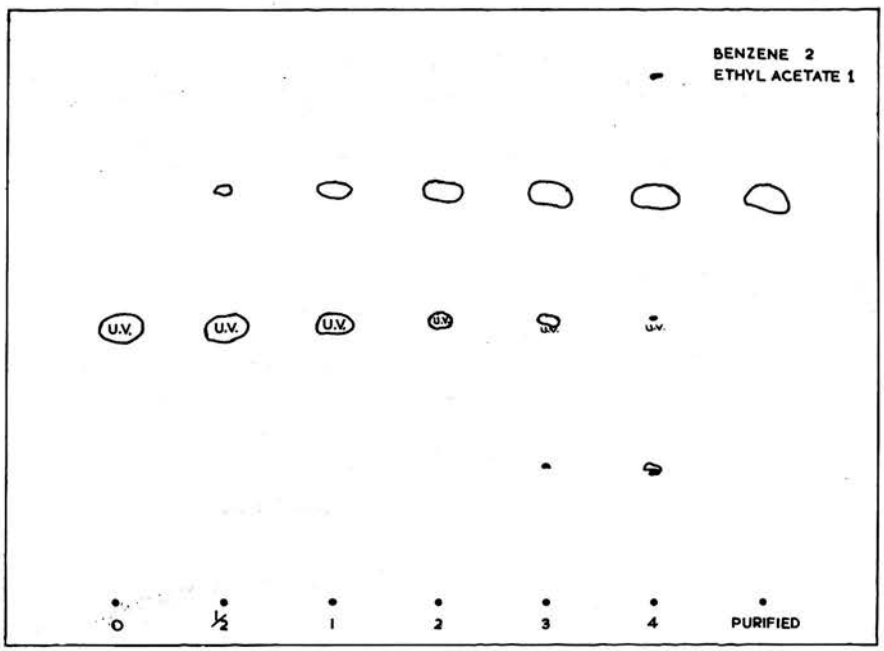

SYNTHESIS OF COPROSTAN—3—ONE—7α—OL.
HYDROGENATION OF CHOLEST—4—EN—3—ONE—7α—OL (Pt/C IN ETHER AT—27°C)
1·5
1·0
0·5
OPTICAL DENSITY AT 242·5mμ
1
2
3
4
5
HOURS.

Figure 21

added and the mixture cooled to -27° and hydrogenated at this temperature. Fixed amounts of the reaction mixture were taken out every hour and a known amount of it was evaporated and dissolved in ethanol and the optical density at 242.5 mμ measured. The other portion of the sample was used for TLC. After 1 hour ~5 mg. of the starting material had disappeared and the TLC showed two spots, one u.v. absorbing corresponding to the starting α,β-unsaturated ketone and the other non-u.v. absorbing corresponding to the saturated compound coprostan-3-one-7α-ol. Such tests every hour showed that the reaction was complete in 5 hours. TLC showed traces of some more polar material also. The results are shown in Fig. 21 and 22. The product was filtered and the filtrate evaporated and the residue used for preliminary chromatography of the product from the more polar compounds.



HYDROGENATION OF CHOLEST-4-EN-3-ONE-7α-OL AT-27°

Figure 22

ii) Preparation

The above hydrogenation experiment was repeated with 50 mg. of the cholest-4-en-3-one-7α-ol and 8 mg. of palladium-on-charcoal in 10 ml. of ether. (-27°) No samples were taken out and the reaction was stopped after 5 hours and the product worked up as above. The residue was chromatographed on neutral alumina (20 g.). Elution with benzene and then 5% ether in benzene eluted some less polar material. Coprostan-3-one-7α-ol was eluted with 20% and then 30% ether in benzene. These eluates were pooled together and evaporated and the residue crystallized from ethanol (38 mg.).

m.p. = 121°C

$[\alpha]_D^{18}$ = +16 $C_{27}H_{46}O_2$ requires C = 80.67%

H = 11.44%

IR (ν) = See Appendix Fig. 10 Found C = 80.84%

H = 11.37%

Coprostan-3α-7α-diol

i) By reduction of coprostan-3-one-7α-ol with lithium aluminium hydride.

Coprostan-3-one-7α-ol (12 mg.) was dissolved in 1 ml. of tetrahydrofuran and added to lithium aluminium hydride (20 mg.) and refluxed for 15 minutes. The product was worked up as described in the procedure for the reduction of epoxide and crystallized from ethyl-acetate.

ii) By direct hydrogenation of cholest-4-en-3-one-7α-ol at room temperature.

Cholest-4-en-3-one-7α-ol (60 mg.) was dissolved in 5 ml. of dry ether and 8 mg. 10% palladium-on-charcoal added and hydrogenated at room temperature for 6 hours. The catalyst was filtered off, washed with warm ether, and the filtrate and washings were evaporated to dryness. Thin-layer chromatography showed one main and three other trace spots but there was no starting material left.

Chromatography on 12.5 g. of 'deactivated' alumina (6% water added to neutral alumina) and elutions with 3% ether in benzene and then 5% ether in benzene gave one spot on thin-layer chromatography. The eluates were evaporated to dryness and the residue crystallized from ethyl-acetate. Recrystallization from acetone-alcohol gave 30 mg. of colourless crystals.

iii) From cholest-4-en-3-one-7α-ol or cholest-4-en-3-one-6α,7α-epoxide through a saturated intermediate.

Cholest-4-en-3-one-7α-ol or the epoxide (15 mg.) was dissolved in dry ether (4 ml.) and 10% palladium-on-charcoal (4 mg.) added and hydrogenated at -27°. After 4 hours the cooling bath was removed and the product hydrogenated at room temperature for another 4 hours. The catalyst was filtered off and washed with warm ether. The ether solution was evaporated and dried which gave a colourless solid residue.

In all the three methods described above the colourless solid residue obtained after the evaporation of the eluates melted at 188-192°, but when it was crystallized from methanol or acetone alcohol the melting point was 78-79° (cf. Bergstrom and Krabisch, 1957). Recrystallizations with ethyl acetate did not change the melting point (78-79°) of this material.

m.p. = 78-79° Mixed m.p. = 78-79°

$[\alpha]_D^{18}$ = +14

IR (ν) = See Appendix Fig. 11

Cholest-4-en-3α, 7α-diol

Reduction of cholest-4-en-3-one-6α, 7α-epoxide

Cholest-4-en-3-one-6α, 7α-epoxide (100 mg.) was reduced with lithium aluminium hydride in tetrahydrofuran as described previously. The crude mixture of diols (predominantly 3α-isomer - shown by TLC) was dissolved in alcohol and treated with digitonin (45% alcoholic solution). The supernatant was extracted with ether. The dried solution of ether was evaporated and the residue crystallized from methanol which gave

colourless crystals (65 mg.).

m.p. = 98°C $C_{27}H_{46}O_2$ requires C = 80.53%

H = 11.44%

$[\alpha]_D^{18}$ = +97 found C = 79.65%

IR (ν) = See Appendix Fig. 8 H = 11.51%

Tritium Labelled Cholest-4-en-3-one-7α-ol

Fifty milligrammes of the compound were randomly labelled with tritium by leaving the compound in contact with tritium for two weeks (Wilzbach method). The labile tritium was removed as usual (p. 55) and the product run on a fluorescent thin-layer chromatogram (benzene:ethyl acetate, 2:1). The chromatogram showed one u.v. absorbing spot. It was cut into ten parts and each was eluted separately and counted. The counting showed three radioactive peaks, one corresponding to cholest-4-en-3-one-7α-ol, the other less polar and the third more polar than this compound.

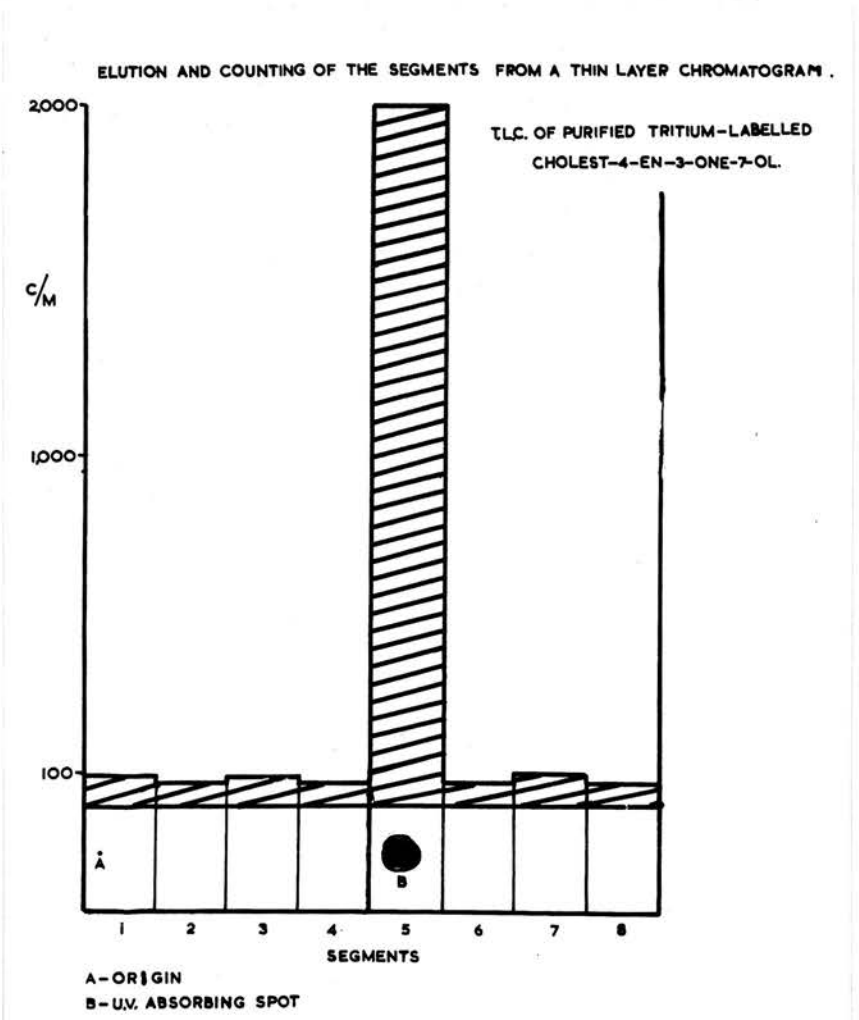

ELUTION AND COUNTING OF THE SEGMENTS FROM A THIN LAYER CHROMATOGRAM.
T.L.C. OF PURIFIED TRITIUM-LABELLED CHOLEST-4-EN-3-ONE-7-OL.
2000
C/M
1,000
100
A
B
1
2
3
4
5
6
7
8
SEGMENTS
A-ORIGIN
B-U.V. ABSORBING SPOT

Figure 23

Several crystallizations with methanol did not improve the quality of the product. The crystalline product was dissolved in 35% ether in petroleum ether (60-80) and chromatographed on 20 g. silicic acid (washed with ether and then methanol and dried at 100° for 24 hours). The column was washed with 35% and then 40% ether in petroleum ether which eluted some less polar material; the cholest-4-en-3-one-7α-ol was eluted (shown by TLC and counting) with 42% and 43% ether in petroleum ether. These cuts were evaporated and the residue crystallized from ethyl acetate to give 12 mg. of the labelled compound (Fig. 23).

m.p. = 183° Specific activity = 7.8×10^6 c.p.m./mg.

Another sample of the compound (30 mg.) was labelled in the same way but giving it only 72 hours' exposure. The product was worked up as usual. It showed very little amounts of some less polar and some more polar material. Chromatography as above gave the pure labelled cholest-4-en-3-one-7α-ol in better yields (18 mg.).

m.p. = 183° Specific activity = 500,000 c.p.m./mg.

Summary

Thin-layer chromatography has been used for the study of a number of steroid reactions.

Coprostan-3α, 7α-diol has been synthesized by a six-stage synthesis from cholesterol. The synthesis proceeds through compounds which have been proposed as intermediates in the biosynthesis of this compound from cholesterol; cholest-4-en-3-one-7α-ol and coprostan-3-one-7α-ol have been synthesized from cholesterol as intermediates in the above synthesis.

The synthesis of two new compounds, cholest-4-en-3α-ol-6α, 7α-epoxide and cholest-4-en-3α, 7α-diol, has been described.

A method of selective reduction of steroid-3-ketones is described. The method of selective reduction of a double bond at reduced temperatures has been applied to cholest-4-en-3-one-7α-ol to synthesize coprostan-3-one-7α-ol.

Cholest-4-en-3-one-7α-ol has been labelled with tritium and purified.

SECTION V

STUDIES ON POSSIBLE INTERMEDIATES INVOLVED IN THE CLEAVAGE OF THE SIDE-CHAIN

Introduction

It has been discussed (p. 20) that in the degradation of cholesterol to bile acids the cleavage of the cholesterol side-chain possibly proceeds through hydroxylated intermediates; 26-, 25-, and 24-hydroxy-cholesterols are possible intermediates. In this work these hydroxylated derivatives have been synthesized by different methods.

Cholest-5-en-3β-26-diol has been prepared from kryptogenin by the method of Scheer et al. (1956). These authors noticed that a Clemmensen reduction of the plant sterol kryptogenin, cholest-5-en-3β-26-diol-16, 22-dione, did not reduce the 16-carbonyl as effectively as it reduced the 22-carbonyl and the reaction product was a mixture of cholest-5-en-3β-26-diol and cholest-5-en-3β-26-diol-16-one. They also reported that if the sterol was subjected to a Huang-Minlon reduction (Huang-Minlon, 1946, 1949) they got a resinous product which could not be crystallized.

In this work these two observations have been confirmed. When kryptogenin was reduced by a Huang-Minlon method the product was a gum which on TLC showed three spots and could not be purified because of its resinous nature. The Clemmensen reduction of the sterol gave a solid colourless precipitate, also giving three or four spots on TLC.

The Clemmensen reduction of kryptogenin was followed every 20 minutes by TLC; a sample was taken out and half of it was poured into cold water and the other half put on to a thin-layer chromatogram. The product, from the first half, was extracted with ether and a spot of this also put on TLC. The developed chromatograms were identical in both cases. The reaction was stopped after 2 hours since the chromatogram showed traces of some new products at this stage. Several methods were used for the isolation of the product of this reduction. The product was either acetylated and the isolation of cholest-5-en-3β-26-diol diacetate effected by chromatography of the diacetates,

or it was subjected to a Huang-Minlon reduction to remove the 16-carbonyl of the unchanged cholest-5-en-3β-26-diol-16-one, or it was reduced with borohydride and chromatographed.

In the chromatography of the acetylated mixture (cholest-5-en-3β-26-diol-diacetate and 3β-26-diacetoxy-cholest-5-en-16-one) on alumina it was noticed that the primary alcoholic acetate at C_{26} readily underwent a hydrolysis to give a free hydroxyl in that position (shown by infrared spectrum and the chromatographic behaviour of the product). The acetate group at C_3 also formed a free hydroxyl but not as readily as the one at C_{26} (cf. Johnsand Jerina, 1963). These products were not isolated pure.

3β-26-Diacetoxy-cholest-5-en-16-one was obtained as a by-product from the chromatography of the diacetates. Some of this product was hydrolysed to get the free keto-diol, cholest-5-en-3β-26-diol-16-one.

In the Clemmensen reduction of kryptogenin in addition to the cholest-5-en-3β-26-diol and the cholest-5-en-3β-26-diol-16-one, another product (compound X) was obtained which could not be identified. Its chromatographic behaviour suggests that it has only one hydroxyl and no other polar substituents; infrared spectrum shows a hydroxyl peak and no carbonyl absorption (Appendix Fig. 16); the compound has a sharp melting point and runs as a single spot on TLC.

Cholest-5-en-3β-26-diol was labelled with tritium by the Wilzbach method (Wilzbach, 1957). Longer exposures with tritium led to several artifacts (shown by TLC and scintillation counting) and the compound could not be purified either by crystallization or by chromatography. The product was then acetylated and the pure tritium-labelled cholest-5-en-3β-26-diol-

diacetate isolated by chromatography on 'neutral' alumina (see Fig. 26 and 27). For the labelling of another batch of cholest-5-en-3β-26-diol, the compound was given a shorter exposure (72 hour) with tritium which did not produce as many artifacts and the compound could then be purified by crystallization only.

For the synthesis of cholest-5-en-3β-25-diol a known method (Ryer, Gebert and Murrill, 1950; Dauben and Bradlow, 1950) was used. 25-Keto-norcholest-5-en-3β-acetate obtained from Steraloids Inc., New York (for the synthesis of this compound see Ruzicka and Fischer, 1937) was treated with methylmagnesium iodide to get the product which was purified by chromatography on alumina.

Cholest-5-en-3β-24-diol has been prepared by a sequence of reactions according to Riegel and Kaye (1944). For the synthesis of this compound 3β-hydroxychol-5-enic acid was prepared from hyodeoxycholic acid (Bharucha et al. 1956). For the preparation of the acyl

chloride of 3β-hydroxy-chol-5-enic acid some workers (Kuwada and Yago, 1937; Cortese and Bauman, 1935) have used thionyl chloride but oxalyl chloride was found to be superior.

Since the acyl chloride is very unstable because of the easily ionizable chlorine atom, the purity of the compound could not be checked by the usual methods, viz. TLC, infrared absorption spectrum, optical rotation, etc. The estimation of the chloride was found to give a satisfactory criteria of the purity of the compound. The acyl chloride 3β-hydroxy-chol-5-enyl-chloride was treated with a Grignard reagent prepared from 2-bromopropane, magnesium and cadmium bromide in ether, and the resulting 3β-acetoxy-cholest-5-en-24-one hydrolysed to get the free hydroxy-ketone or was reduced with a metal hydride reagent to give the diol which was purified by chromatography (Fig 2).

Cholest-5-en-3β-24-diol was also prepared by the ozonolysis of fucosterol. Fucosterol is a 5, 6-unsaturated C_{29} plant sterol having a $=CH.CH_3$ group

attached at C_{24}. The rest of the molecule is like cholesterol. This sterol has been found in all species of the brown marine algae and can be extracted easily from Fucus Vesiculosus. Since the molecule is like cholesterol and it has a $=CH.CH_3$ group attached at C_{24}, it will be expected to give 24-ketocholesterol on ozonolysis. Fucosteryl acetate was ozonized by a slow stream of the gas at room temperature (Hey, Honeyman and Peal, 1950). The hydrolysed product was found to be identical with cholest-5-en-3β-ol-24-one prepared by the previous method. Reduction of this hydroxy ketone with lithium aluminium hydride gave the diol (Fig. 28).

As pointed out in the introduction, the 3α, 7α, 12α-trihydroxy coprostanic compounds having oxygen functions at C_{24} have been shown to convert into cholic acid less efficiently than the corresponding compounds without an oxygen function at C_{24} (p. 27). Such studies with 3α, 7α-dihydroxy-24-oxo compounds have not been undertaken because no such compounds have yet been synthesized.

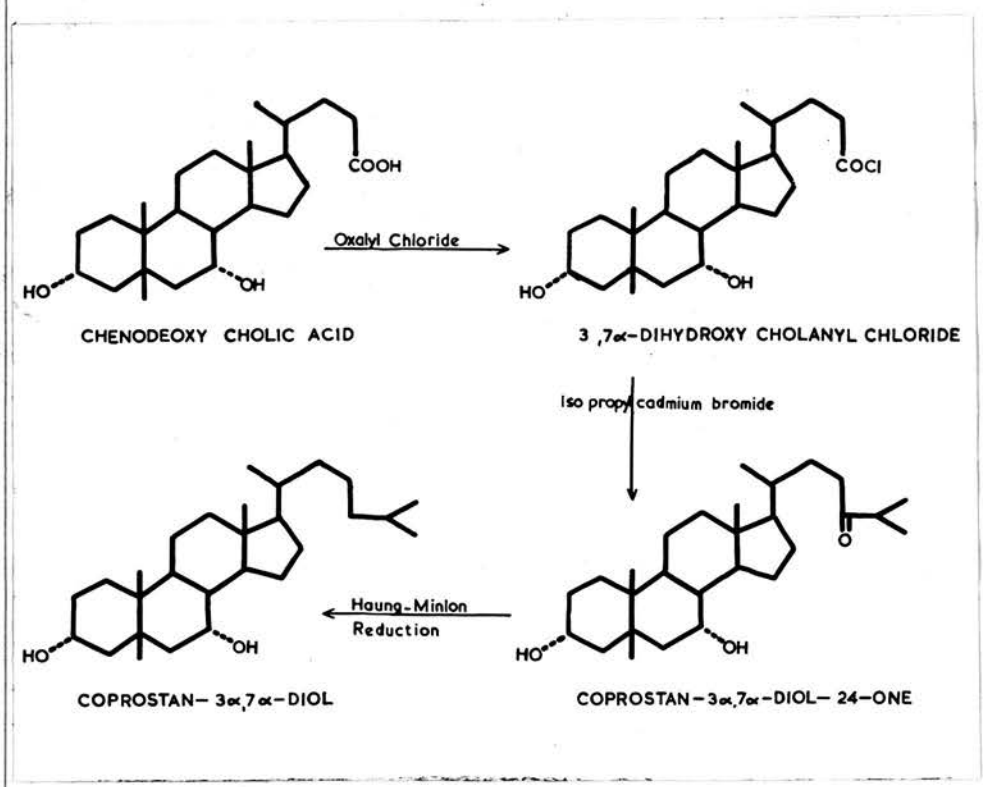

COOH
HO
OH
Oxalyl Chloride
COCl
HO
OH
CHENODEOXY CHOLIC ACID
3 ,7α-DIHYDROXY CHOLANYL CHLORIDE
Iso propyl cadmium bromide
O
HO
OH
Haung-Minlon
Reduction
HO
OH
COPROSTAN- 3α,7α-DIOL
COPROSTAN-3α,7α-DIOL- 24-ONE

Figure 24

Cole and Julian (1945) and Shimizu, Noda and Yamasaki (1958) evolved a method of preparing steroids with a keto group in the side-chain. Applying this method, coprostan-3α, 7α-diol-24-one has been synthesized by a series of reactions (Fig 24)

3α, 7α-Dihydroxy-cholanic acid (chenodeoxycholic acid) was prepared from cholic acid by a selective hydrolysis of 3α, 7α, 12α-triacetoxy-cholanic acid at C_{12} and the subsequent oxidation at this position (C_{12}) and then removal of the 12-carbonyl by a Huang-Minlon reduction (Hoffman, 1963; Fieser and Rajagopalan Bhattacharya, 1950). The pure chenodeoxycholic acid was acetylated to get the 3α, 7α-diacetoxy-cholanic acid which gave 3α, 7α-diacetoxy-cholanyl chloride by treatment with oxalyl chloride. This acyl chloride gave a correct value for the chloride and that was taken as a criteria of its purity. 3α, 7α-Diacetoxy-coprostan-24-one was obtained by a Grignard reaction with 3α, 7α-diacetoxy-cholanyl chloride and 2-bromopropane. The reaction product was hydrolysed and 3α, 7α-dihydroxy-coprostan-24-one isolated pure. Several solvents were tried to crystallize this product but it gave a gum which did not crystallize. The

solvent was then evaporated and the product dissolved in ether and evaporated. This treatment when repeated three times gave a dry solid residue which was crushed to a powder. It gives a single spot on TLC in two different solvent systems. Its infrared absorption spectrum shows a hydroxyl and a side-chain ketone (Appendix Fig. 32).

It will be of considerable interest to test this compound and the corresponding 3, 24-diol, coprostan-3, 24-diol, on a bile fistula animal and see if it is converted into chenodeoxycholic acid (cf. p. 27), Shimizu et al. 1959).

Methods of Synthesis

Cholest-5-en-3β, 26-diol-16, 22-dione (kryptogenin)

The kryptogenin obtained from Steraloids Inc., U.S.A., was found to contain several other products when tested on thin-layer chromatography. Infrared spectrum and melting point (159°C; lit. 181°) were also unsatisfactory.

The crude material was leached several times with cold ether and the solid crystallized from ethyl acetate. Two crystallizations removed some impurities but the crystals still showed traces of less polar material (TLC - solvent system, benzene:ethyl acetate:acetone, 10:5:5). The crystals were dissolved in 10% methanol in chloroform and chromatographed on 'active' alumina. Elution with 20% methanol in chloroform eluted the less polar material and eluates from 50% methanol in chloroform and then 100% methanol gave kryptogenin. Crystallization from ethyl acetate afforded colourless

needles.

m.p. = 183° C & H ($C_{27}H_{40}O_4$) Theory C = 75.3% H = 9.7% Found C = 75.1% H = 9.6%

IR (ν) Impure sample = See Appendix Fig. 13

Purified sample = See Appendix Fig. 14

A further crop of pure material was obtained when the ether extracts and the mother liquors from different crystallizations were pooled together, evaporated and chromatographed.

A 20 g. batch of kryptogenin obtained from Lights (L. Light and Co., Ltd., England) was found to be of a better quality but showed traces of two less polar products which were removed by two crystallizations with ethyl acetate and the crystalline product gave a single spot on TLC.

m.p. = 183°

Cholest-5-en-3β, 26-diol (26-hydroxycholesterol)

Clemmensen Reduction of Kryptogenin

Zinc amalgam (40 g.) (prepared by shaking for 10 minutes 50 g. of powdered zinc and 6 g. of mercuric

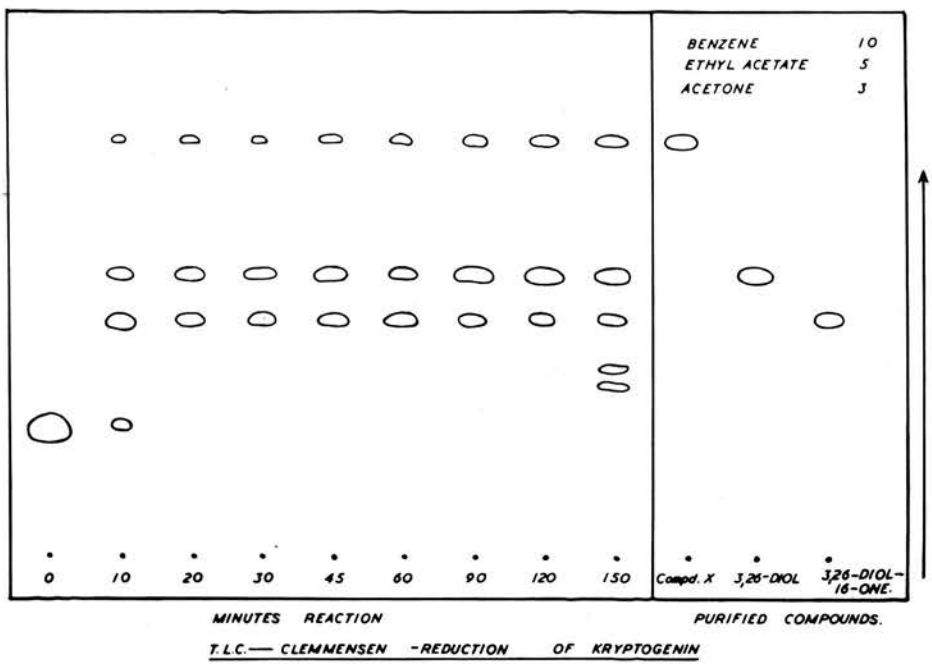


Figure 25

chloride in 100 ml. of water and 3 ml. of concentrated HCl) was taken in a 500 ml. R. B. flask fitted with a condenser. Kryptogenin (1.5 g.) was dissolved in 150 ml. of 95% ethanol and added to the flask and the mixture heated to reflux; 60 ml. of concentrated HCl were added in small portions over a period of 2 hours and the mixture kept refluxing for another 30 minutes. The reaction mixture was sampled out every 20 minutes and run on TLC (benzene:ethyl acetate, 2:1). The starting material disappeared during the first 20 minutes and the product showed two main spots, one corresponding to the cholest-5-en-3β, 26-diol and the other slightly more polar presumably cholest-5-en-3β,-26-diol-16-one. In the subsequent TLC this latter compound seemed to decrease while 26-hydroxycholesterol was increasing. The chromatograms also showed a spot (R_F 0.78) less polar than 26-hydroxycholesterol. After 2 hours the chromatograms showed two more

polar spots and the reaction was stopped at this stage (Fig 25); the reaction mixture was allowed to cool and was then poured into cold water and the product extracted with ether, evaporated and dried.

TLC of the dried product showed two main spots and one small less polar one and two trace spots of more polar material. Two crystallizations from ethyl acetate removed the small spots and the crystals (0.82 g.) showed only two spots corresponding to the diol and the keto-diol. Further crystallization did not separate the two compounds. The 26-hydroxycholesterol was isolated from the mixture by chromatography of the diacetates.

Compound 'X'

The supernatant from the above ethyl acetate crystallization was evaporated to dryness and re-crystallized from ethyl acetate which gave a product 'X' running as a single spot on TLC (R_F = 0.78; benzene:ethyl acetate, 2:1). The chromatographic

behaviour suggests one nuclear hydroxyl group; it gives a single spot on TLC in two different solvent systems and has a sharp melting point. The infrared spectrum shows a hydroxyl peak but no carbonyl.

m.p. = 116°	C & H	Theory	Found
		C = ?	C = 78·56%
IR (ν) = See Appendix Fig. 16		H = ?	H = 9·02%

Isolation of Cholest-5-en-3β, 26-diol

The isolation of the cholest-5-en-3β, 26-diol from the Clemmensen reduction product was effected by different methods.

i) By chromatography of the diacetates

Acetylation of the mixture

The crystalline product (mixture of cholest-5-en-3β, 26-diol and cholest-5-en-3β, 26-diol-16-one) obtained above was refluxed with acetic anhydride for 1 hour, allowed to cool and then poured slowly into crushed ice and the product extracted with ether, washed, dried and evaporated.

Cholest-5-en-3β, 26-diol-diacetate

The solid residue obtained above (0.75 g.) was dissolved in benzene and chromatographed on 'neutral' alumina (30 g.). Elution with 0.5% ethyl acetate in benzene gave a product which gave one spot on TLC (benzene:petroleum ether:ethyl acetate, 40:60:5). These eluates were pooled together, evaporated and crystallized from methanol which gave cholest-5-en-3β, 26-diol-diacetate as shining needles (0.355 g.).

m.p. = 119° C & H ($C_{31}H_{50}O_4$)

$[\alpha]_D^{18}$ = +11 Theory C = 76.6%

IR (ν) = See Appendix Fig. 17 H = 10.37%

Found C = 76.64%

H = 10.53%

Cholest-5-en-3β, 26-diol (26-hydroxycholesterol)

The above diacetate (200 mg.) was hydrolysed by refluxing with 30 ml. 5% methanolic KOH for 1 hour. The product was extracted with ether, washed, dried, evaporated and crystallized from ethyl acetate to give

the free compound, cholest-5-en-3β, 26-diol (0.165 g.).

m.p. = 178° C & H ($C_{27}H_{46}O_2$)

$[\alpha]_D^{18}$ = -30 Theory C = 80.6%

IR (ν) = See Appendix Fig. 18 H = 11.4%

Found C = 80.5%

H = 11.3%

3β, 26-Diacetoxy-cholest-5-en-16-one

Continued elution of the above column (chromatography of the diacetates) with 5% ethyl acetate in benzene gave a product which gave one spot on TLC (benzene:petroleum ether:ethyl acetate, 40:60:5). The eluates were evaporated and crystallized from methanol to give shining needles of 3β, 26-diacetoxy-cholest-5-en-16-one (0.22 g.).

m.p. = 116° C & H ($C_{31}H_{48}O_5$)

$[\alpha]_D^{18}$ = -118 Theory C = 77.8%

IR (ν) = See Appendix Fig. 19 H = 10.5%

Found C = 77.6%

H = 10.6%

Cholest-5-en-3β, 26-diol-16-one

The compound (35 mg.) was obtained by the hydrolysis of the above diacetate (50 mg.).

m.p. = 171°

$[\alpha]_D^{18}$ = -155

Further elution of the above column (chromatography of diacetates) with 10% and then 25% ethyl acetate in benzene eluted a product which gave two spots on TLC (benzene:ethyl acetate, 2:1). The mobility of these compounds suggests that the less polar one (R_F 0.8) has one hydroxyl group and the more polar one (R_F 0.58) has a ketone in addition. The infrared spectrum of this product shows a hydroxyl as well as a ketone absorption (see Appendix Fig. 20).

These two products seem to have formed by the hydrolysis of the primary alcoholic acetate group at C_{26} (cf. Johns and Jerina, 1963). The two products were not separated for any further investigation. Forty percent

and 50% ethyl acetate eluted two more polar products which behaved like cholest-5-en-3β, 26-diol and cholest-5-en-3β, 26-diol-16-one. The infrared spectrum shows a carbonyl and a hydroxyl absorption. These compounds seem to have been formed by the complete hydrolysis of the diacetates on the alumina column (cf. Johnsand Jerina, 1963).

All these eluates (10% to 50% ethyl acetate in benzene) were pooled together and hydrolysed with KOH. The product of hydrolysis showed two spots on TLC (benzene:ethyl acetate:acetone, 10:5:3) corresponding to cholest-5-en-3β, 26-diol and cholest-5-en-3β, 26-diol-16-one (0.090 g.). This shows that the two less polar products obtained above (eluates from 10% and 25% ethyl acetate in benzene) were, indeed, products of partial hydrolysis of the diacetates.

ii) By Huang-Minlon reduction

Kryptogenin (1.8 g.) was reduced first by a Clemmensen method. The mixture of cholest-5-en-3β, 26-diol and 16-keto-cholest-5-en-3β, 26-diol

obtained by this Clemmensen reduction of kryptogenin was mixed with the same mixture obtained from the chromatography of the diacetates (after saponification of the residue from ethyl acetate and methanol eluates, p. 136, and was subjected to a Huang-Minlon reduction (Huang-Minlon, 1949).

The above mixture (0.75 g.), 15 ml. of triethylene glycol, 1.5 ml. of hydrazine hydrate and 1.3 g. of KOH were heated at 150-165° on a sand bath in a flask fitted with an air condenser. The condenser was removed after 2 hours and the temperature allowed to rise to 185° and kept there for 1 hour. The reaction mixture was allowed to cool and then poured into cold 1N-HCl while stirring. The precipitate was filtered, washed and crystallized from ethyl acetate to give cholest-5-en-3β, 26-diol.

m.p. = 178°

$[\alpha]_D^{18}$ = -30

iii) By reduction with sodium borohydride

The mixture (250 mg.) was dissolved in 10 ml. of methanol and added to a methanolic solution (10 ml.) of sodium borohydride (250 mg.) and the solution left at room temperature for 1 hour. The reagent was decomposed with water and the product extracted with ether-methanol. The extracts were dried and evaporated and the residue chromatographed on alumina (Peter Spence type 'O'). Elution with 5% and then 10% methanol in ether eluted the 26-hydroxycholesterol. These eluates were pooled together, evaporated and crystallized from ethyl acetate.

m.p. = 178

$[\alpha]_D^{18}$ = -30

That the products obtained by the three methods described above were identical was shown by mixed melting point. There was no depression.

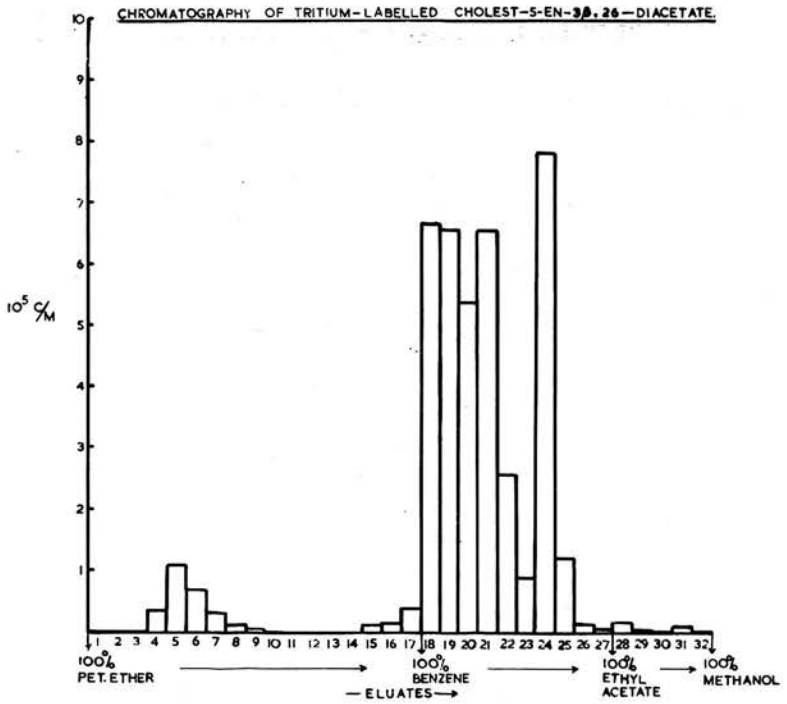

CHROMATOGRAPHY OF TRITIUM-LABELLED CHOLEST-5-EN-3β, 26-DIACETATE.
10⁵ C/M
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32
100% PET. ETHER
100% BENZENE
100% ETHYL ACETATE
100% METHANOL
— ELUATES →

Figure 26

Tritium Labelled Cholest-5-en-3β, 26-diol

The compound (75 mg.) was left exposed with tritium for two weeks. The labile tritium was removed in the usual way. TLC of the product at this stage showed three radioactive peaks. Chromatography on alumina or silicic acid and repeated crystallizations did not improve the quality of the product. The dry product was treated with acetic anhydride to get the diacetate which was chromatographed. The product was dissolved in petroleum ether and adsorbed on neutral alumina. Increasing amounts of benzene in petroleum ether eluted one radioactive peak in the eluates from 20% to 30% benzene in petroleum ether. The cholest-5-en-3β, 26-diol-diacetate was eluted with benzene and then 0.5% ethyl acetate and 2% ethyl acetate in benzene. Continued elution with increasing amounts of ethyl acetate in benzene eluted some more polar material. The results are shown in the form of a graph (Fig. 26 and 27).

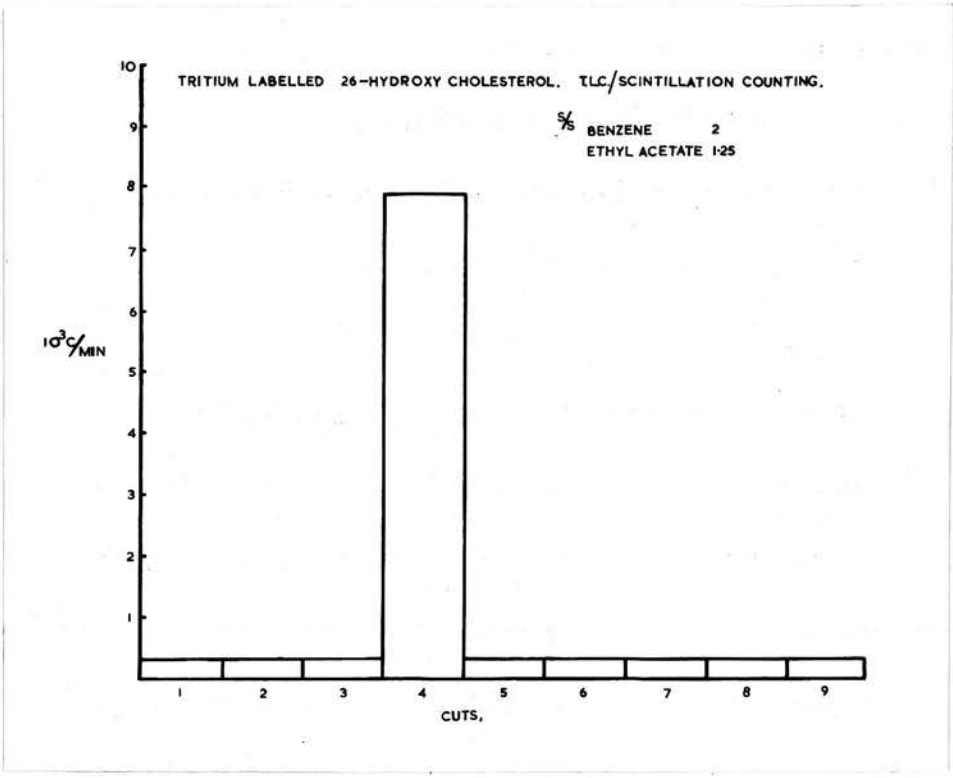

TRITIUM LABELLED 26-HYDROXY CHOLESTEROL. TLC/SCINTILLATION COUNTING.
S/S BENZENE 2
ETHYL ACETATE 1·25
10
9
8
7
6
5
4
3
2
1
10^3C/MIN
1
2
3
4
5
6
7
8
9
CUTS.

Figure 27

The cuts consisting of the cholest-5-en-3β, 26-diol-diacetate were pooled together, evaporated and the residue crystallized from methanol.

The diacetate was hydrolysed with methanolic KOH to give the free compound (17 mg.).

m.p. = 178°

Specific activity = 8.96×10^6 c.p.m./mg.

In another run, 30 mg. of the compound were left in contact with tritium for only 72 hours. The product in this case showed very little of the artifacts and was sufficiently radioactive. After removal of the labile tritium in the usual way the product was crystallized three times with ethyl acetate and once with methanol which gave a pure product.

m.p. = 178°

Specific activity = 5.6×10^4 c.p.m./mg.

Cholest-5-en-3β, 25-diol (25-hydroxycholesterol)

The compound was prepared by a Grignard reaction with methyl magnesium iodide and 25-keto-nor-cholest-5-en-3β-ol-acetate. The latter compound was obtained from Steraloids Inc., New York (m.p. = 141-144°; for infrared spectrum see Appendix Fig. 21).

A Grignard reagent was prepared from methyl iodide and magnesium in ether. Dry magnesium (0.20 g.) was dissolved in 1 ml. (1.13 g.) of methyl iodide in 10 ml. of ether. A benzene solution of the steroid (500 mg. in 6 ml. of benzene) was added to the reagent and the mixture refluxed for 3 hours and then left at room temperature overnight. Ice water (30 ml.) and 50% acetic acid (2 ml.) was added and the mixture steam-distilled for about 30 minutes until no more oil passed over; the product precipitated out as colourless flakes. The precipitate was filtered, washed and dried. TLC (benzene:ethyl acetate, 2:1) at this stage showed traces

of some less polar material (R_F = 0.85; R_F of the diol = 0.5). The dried precipitate was crystallized from methanol but the crystals still showed some less polar material. The dried crystals were dissolved in 75% ether in benzene and chromatographed on 'active' alumina (p. 43). Seventy-five percent ether in benzene and then ether eluted the less polar material and the cholest-5-en-3β, 25-diol was eluted with 5% and then 10% methanol in ether. Crystallization of these eluates from methanol gave colourless crystals (540 mg.).

m.p. = 182°

$[\alpha]_D^{18}$ = -37°

IR (ν) = See Appendix Fig. 22

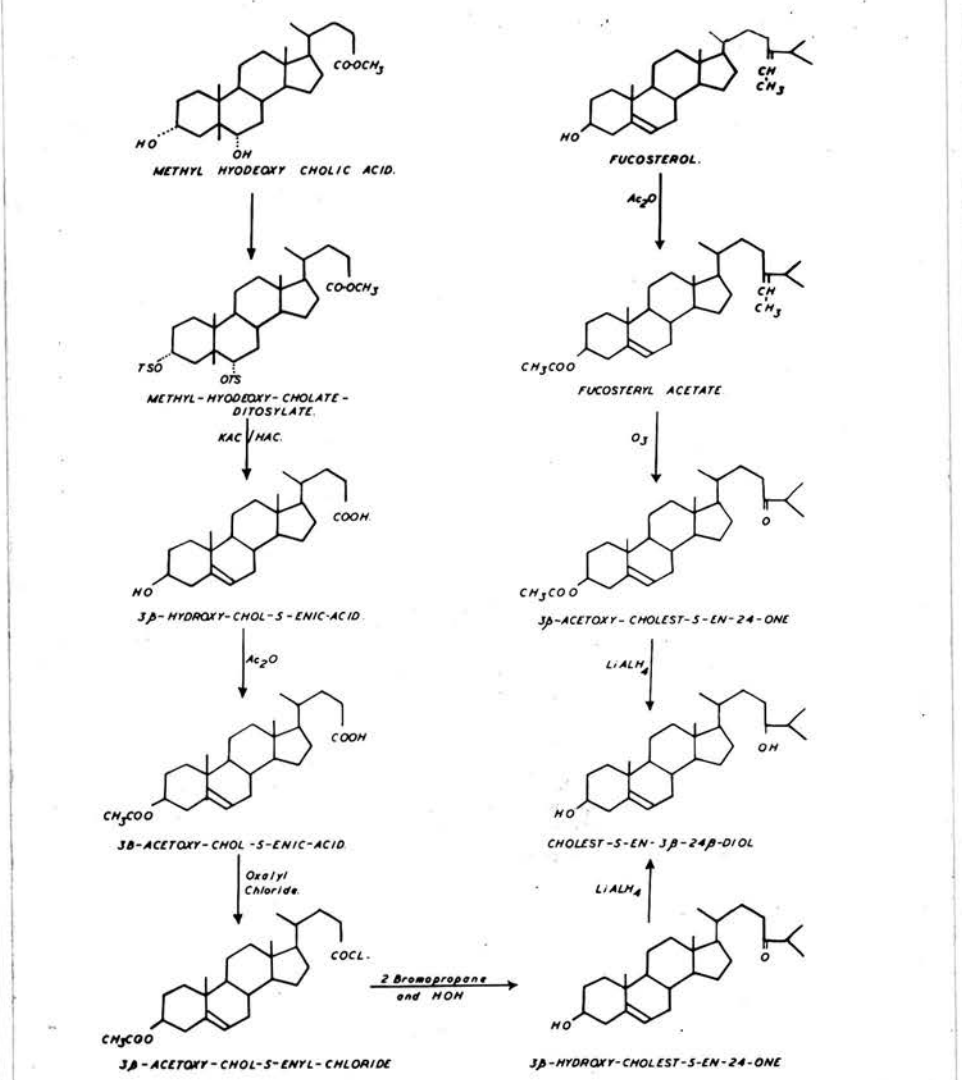
C& H ($C_{27}H_{46}O_2$)

Theory C = 80.5%

H = 11.4%

Found C = 80.01%

H = 11.6%



ROUTES FOR THE SYNTHESIS OF CHOLEST-5-EN-3β-24β-DIOL

Figure 28

Cholest-5-en-3β, 24-diol

3β-Acetoxy-Δ^5-cholenyl chloride

Methyl Hyodeoxycholate

Hyodeoxycholic acid (25 g.) was dissolved in methanol (500 ml.) and concentrated HCl (20 ml.) added and the mixture refluxed for 3 hours. The product was allowed to cool and then poured, with constant stirring, into cold 1N-HCl containing chipped ice. The precipitate was allowed to settle overnight, filtered, thoroughly washed, dried and crystallized from methanol. It showed one spot on TLC (benzene:ethyl acetate:acetone, 10:5:3).

C & H $(C_{25}H_{41}O_4)$

Theory C = 74.07%

H = 10.1%

Found C = 73.9%

H = 10.4%

Methyl Hyodeoxycholate Ditosylate

To 25 g. of methyl hyodeoxycholate in 50 ml. of dry pyridine, 28.8 g. of p-toluene sulphonyl chloride was added at 0° and then left at room temperature. After

48 hours the excess reagent was decomposed with ice and the mixture poured into ice cold 1N-HCl. The dried ether extract was evaporated and the residue crystallized from ethyl acetate.

m.p. = 162-164°

$[\alpha]_D^{18}$ = +7

IR (ν) = See Appendix Fig. 23

3β-Hydroxy-chol-5-enic Acid

Potassium acetate (7.5 g.) was taken in 3.5 ml. of water and 40 ml. of dimethyl formamide and 2 ml. of acetic acid added and the mixture heated at 100-105° for 30 minutes. Methyl hyodeoxycholate ditosylate (5 g.) was then added and the mixture heated at 100° for another 5 hours. The cooled reaction mixture was poured into ice cold 1N-HCl and the precipitate dissolved in ether and washed. The dried ether extract was evaporated and the residue crystallized from ethyl acetate and then acetic acid. The product gave one spot on TLC (benzene: ethyl acetate:acetone, 10:5:5).

m.p. = 228-232° C & H ($C_{24}H_{38}O_3$)

IR (ν) = See Appendix Fig. 24 Theory C = 76.9%

H = 10.1%

Found C = 76.53%

H = 9.98%

3β-Acetoxy-chol-5-enic Acid

3β-Hydroxy-chol-5-enic acid (5 g.), acetic anhydride (25 ml.) and pyridine (10 ml.) were shaken and left at room temperature overnight. The product was slowly poured into ice and extracted with ether, the ether extracts washed, dried and evaporated and the residue crystallized from methanol having a trace of ether to get pure compound. The crystalline product runs as a single spot on TLC (benzene:ethyl acetate, 2:1).

m.p. = 152-153° C & H ($C_{26}H_{40}O_4$)

IR (ν) = See Appendix Fig. 25 Theory C = 75.1%

H = 9.4%

Found C = 74.44%

H = 9.52%

3β-Acetoxy-chol-5-enic-chloride

3β-Acetoxy-chol-5-enic acid (5 g.) was dissolved in 5 ml. oxalyl chloride and left at room temperature for 2 hours. Benzene-ether (1:1) (25 ml.) was then added and the solvents evaporated at reduced pressure. The process was repeated three times which gave a residue of white powdery mass. This was dried under reduced pressure for about 2 hours. The residue gave a correct elemental analysis for the halogen and was used for the next step without further purification.

$C_{26}H_{37}O_3Cl$ required Cl = 8.3%

Found Cl = 7.9%.

Cholest-5-en-3β-ol-24-one

i) By Grignard reaction of 3β-acetoxy-chol-5-enyl chloride with 2-bromopropane.

Magnesium turnings (0.600 g.) were gradually dissolved in a mixture of ether (25 ml.) and 2-bromopropane (3 ml. ≡ 3.6 g.) by shaking vigorously.

Anhydrous cadmium bromide (7.5 g.) was then added slowly while the mixture was kept shaken and cool. To this Grignard reagent was added a benzene solution (7 ml.) of 3β-acetoxy-chol-5-enyl-chloride (1.5 g.). The mixture was refluxed for 1 hour and then left at room temperature overnight. Crushed ice and then cold 3N-HCl was added and the ether-benzene layer separated. The aqueous layer was extracted with more ether-benzene. The extracts were washed thoroughly with water, dried and evaporated. The residue was crystallized twice from ethyl acetate and then with methanol which gave needle-like crystals of 3β-acetoxy-cholest-5-en-24-one.

m.p. = 127° C & H ($C_{29}H_{46}O_3$)

IR (ν) = See Appendix Fig. 26

Theory C = 78.7%
H = 10.4%

Found C = 78.11%
H = 10.30%

3β-Acetoxy-cholest-5-en-24-one (0.48 g.) was saponified with 5% methanolic KOH and the product extracted with ether. The ether extract was evaporated and the residue crystallized from petroleum ether to give cholest-5-en-3β-ol-24-one as needles.

m.p. = 135°

$[\alpha]_D^{18}$ = -35

IR (ν) = See Appendix Fig. 27

(ii) By ozonolysis of fucosterol

Fucosterol

Fucosterol obtained from Mr E. Booth of the Arthur D. Little Research Institute, Musselburgh (see also p. 124) was found to contain several other products when tested on TLC (benzene:ethyl acetate, 2:1). Besides fucosterol (R_F = 0.7) the chromatogram showed several other components more polar than fucosterol. The compound (2 g.) was dissolved in 25% ether in benzene which left some insoluble gum weighing about one gram. That this gum did not contain any fucosterol was shown by TLC of a chloroform solution of the gum.

The above ether-benzene (1:3) solution was chromatographed on 'active' alumina (p. 43). The column was washed with 25% and then 30% ether in benzene. Fucosterol was eluted with 50% and then 60% ether in benzene (500 ml. each). Seventy percent and 80% ether in benzene eluted some more polar material. The eluates containing only fucosterol were pooled and evaporated and the residue crystallized from methanol (760 mg.).

m.p. = 121° C & H ($C_{29}H_{48}O$)

IR (ν) = See Appendix Fig. 28

Theory C = 84.4%
H = 11.6%
Found C = 84.41%
H = 11.76%

Fucosteryl Acetate

Fucosterol (500 mg.) was refluxed with acetic anhydride (5 ml.) for 1 hour. The product was poured slowly into a chilled beaker; the acetate crystallized.

The crystals were washed with chilled methanol and recrystallized from ether-methanol (1:9) which gave 495 mg. of the product as shining needles.

m.p. = 119° C & H ($C_{31}H_{50}O_2$)

IR (ν) = See Appendix Fig. 29

Theory C = 81.9%
H = 11.01%

Found C = 82.06%
H = 10.95%

Cholest-5-en-3β-ol-24-one acetate

Fucosteryl acetate (250 mg.) was taken in 10 ml. of glacial acetic acid and ozone (about 1%) passed through the suspension for 4 hours. A sample of the reaction mixture was analysed by TLC every half an hour. After the first 30 minutes the product showed a more polar spot in addition to the starting material. This spot correspondingly increased and after 4 hours there was no trace of the starting material left. The ozonization was then stopped and 10 ml. of water added and the reaction mixture boiled until free from acetaldehyde. The product was extracted with chloro-

form, washed and dried. Evaporation of the extracts and crystallization of the residue from methanol gave 3β-acetoxy-cholest-5-en-24-one as needles (180 mg.).

m.p. = 127-128°

$[\alpha]_D^{18}$ = -42

IR (ν) = See Appendix Fig. 30

C & H ($C_{29}H_{46}O_3$)

Theory C = 78.7%

H = 10.4%

Found C = 78.11%

H = 10.30%

Cholest-5-en-3β, 24β-diol

i) **From cholest-5-en-3β-ol-24-one**

Cholest-5-en-3β-ol-24-one (200 mg.) was dissolved in 5 ml. of dry ether and added to a suspension of 200 mg. of lithium aluminium hydride in 5 ml. of ether. The mixture was refluxed for 30 minutes and then allowed to cool. The excess reagent was decomposed with 15 ml. of ether-ethyl acetate (2:1) and the produce worked up as usual (p. 107). The product showed two main spots on TLC, one corresponding to an authentic sample of cholest-5-en-3β, 24β-diol and the other less polar.

Repeated crystallization with ethyl acetate and then methanol gave pure cholest-5-en-3β, 24β-diol.

m.p. = 174°

ii) From 3β-acetoxy-cholest-5-en-24-one (from fucosterol)

3β-Acetoxy-cholest-5-en-24-one (200 mg.) was reduced with lithium aluminium hydride and the product worked up as above to give pure cholest-5-en-3β, 24β-diol.

m.p. = 176° I.R. = Appendix Fig 30.

$[\alpha]_D^{18}$ = -46

Coprostan-3α, 7α-diol-24-one

3α, 7α-Dihydroxy-cholanic acid (chenodeoxycholic acid)

The compound was prepared from cholic acid by the method of Fieser and Rajagopalan (1950) according to Hoffmann (1963).

m.p. = 137-139°

$[\alpha]_D^{18}$ = +9

IR (ν) = See Appendix Fig. 33

3α, 7α-Diacetoxy-5β-cholanic acid

3α, 7α-Dihydroxy-5β-cholanic acid (5 g.) was acetylated with acetic anhydride (10 ml.) and pyridine (2 ml.) at room temperature. The product was poured into ice, extracted with ether, washed, dried, evaporated and crystallized from methanol-ether (9:1) which gave needle-like crystals (4.9 g.). The compound is acidic and runs as a single spot on TLC (benzene: ethyl acetate, 2:1).

m.p. = 118-122°

IR (ν) = See Appendix Fig. 31

$C_{28}H_{44}O_6$ requires C = 70.58%

H = 9.24%

Found C = 70.08%

H = 9.05%

3α, 7α-Diacetoxy-cholanyl chloride

3α, 7α-Diacetoxy-cholanic acid (3.5 g.) **was treated with oxalyl chloride** (7 ml.) **for 2 hours. The product was worked up as usual. The white powdery mass was used for the next step.**

Required Cl = 8.7%

Found = 7.9%

3α, 7α-Dihydroxy-coprostan-24-one

A Grignard reagent in ether was prepared from 2-bromo-propane (8 ml.; 9.6 g.), magnesium turnings (1.5 g.), ether (70 ml.) and cadmium bromide (20 g.) as usual (p. 144). **3α, 7α-Dihydroxy-cholanyl chloride (3.0 g.) in benzene** (6 ml.) **was added to the Grignard reagent and the mixture refluxed for 1 hour and left at room temperature overnight. It was worked up as usual** (p. 145) **and the product hydrolysed with methanolic KOH (35 ml. of 5% methanolic KOH solution)**

by refluxing for 1 hour. The hydrolysed product was extracted with ether and the dried extract evaporated to dryness. The product at this stage showed two less polar materials and a trace of some more polar substance on TLC (benzene:ethyl acetate:acetone, 10:5:3). It was dissolved in benzene and chromatographed on 'neutral' alumina (p. 43). The column was washed first with petroleum ether and the compound eluted with 10% benzene and then 20% benzene in petroleum ether. The two eluates gave one spot on TLC (R_F = 0·35) benzene:ethyl acetate:acetone, 10:5:3). The eluates were evaporated to dryness. Different solvents were tried for crystallization but the compound could not be crystallized. It always gave a gummy residue. The residue was then dissolved in ether and evaporated. This process when repeated three times gave a powdery mass which was shown to have one component on TLC; the infrared spectrum has clear peaks and shows hydroxyl and carbonyl absorption.

m.p. = 74-76°

IR (ν) = See Appendix Fig. 32

$C_{27}H_{46}O_3$ requires C = 77.5%

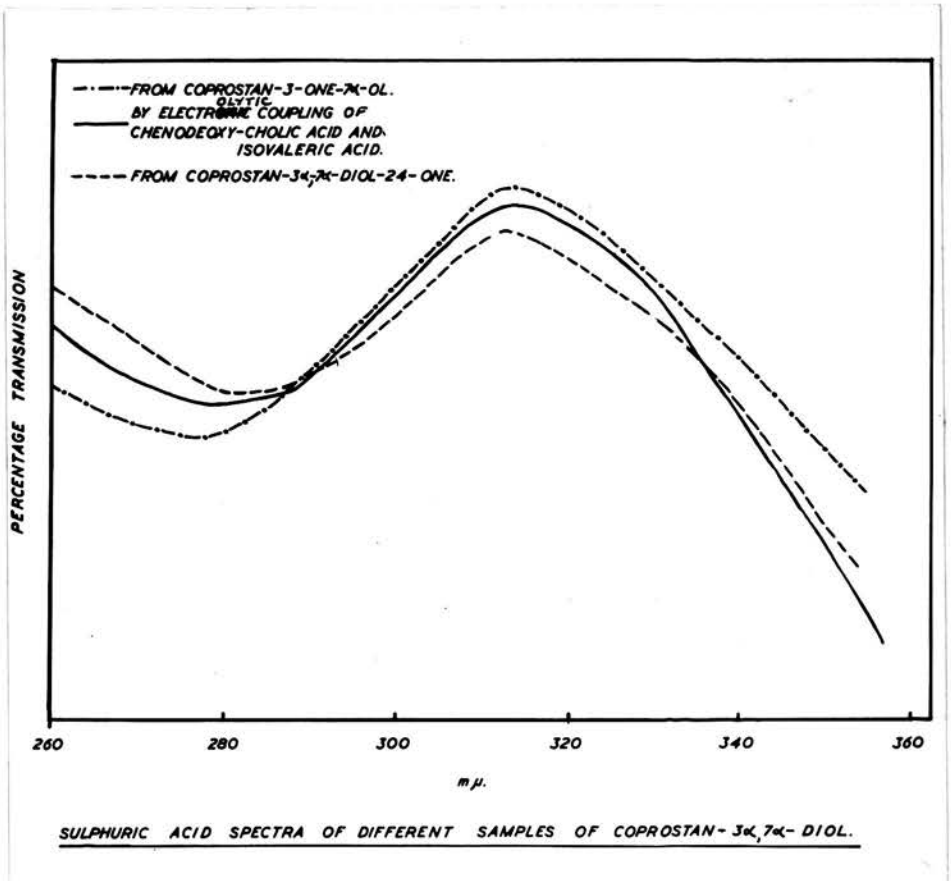
H = 11.0%

Found C = 76·61 %

H = 10·98 %

Coprostan-3α, 7α-diol

3α, 7α-Dihydroxy-coprostan-24-one (0.5 g.), triethylene glycol (10 ml.), hydrazine hydrate 95% (1.0 ml.) and potassium hydroxide pellets (0.8 g.) were refluxed gently at 150° for 45 minutes. The condenser was removed and the solvent allowed to evaporate until the temperature reached 195°. An air-condenser was then fitted and the reaction mixture heated at 195° for 2 hours. The cooled reaction mixture was poured into 0.5N-HCl (75 ml.) with constant stirring. The resulting precipitate was extracted with ether, washed, dried and evaporated. The residue showed a trace of the starting material on TLC (benzene:ethyl acetate: acetone, 10:5:3) which was removed by crystallization of the product with petroleum ether-ethyl acetate. The product came down as a gummy residue while the trace of the impurity stayed in the supernatant (shown by TLC of the supernatant and the residue). Several attempts at the crystallization gave solid plate-like crystals (m.p. = 78-79). The melting point showed



SULPHURIC ACID SPECTRA OF DIFFERENT SAMPLES OF COPROSTAN-3α,7α-DIOL.

Figure 29

no depression on admixture with an authentic sample prepared by other methods (p. 114).

m.p. = 78-79° Mixed m.p. = 78-79°

$[\alpha]_D^{18}$ = 13°

IR (ν) = See Appendix Fig. 11

The sulphuric acid spectra of the three samples of coprostan-3α, 7α-diol obtained by different methods are found to be identical (Fig. 29).

Summary

A number of compounds hydroxylated in the side-chain have been synthesized; 26-, 25-, and 24-hydroxy-cholesterols have been obtained by different methods.

Coprostan-3α, 7α-diol-24-one, a compound which could give considerable information about the mechanism of the cleavage of the side-chain has been synthesized.

Cholest-5-en-3β, 26-diol has been labelled with tritium.

Thin-layer chromatography has been used for following most of the reactions.

SECTION VI

DISCUSSION

The progress achieved in this work is briefly summarized in the following paragraphs.

i) A method has been developed whereby using small quantities of reactants a quantitative study of some steroid reactions can be made.

ii) A method of 7α-hydroxylation of 5, 6-unsaturated steroids has been developed and using this method some possible 7α-hydroxylated intermediates have been synthesized. The syntheses proceed through hydroperoxide intermediates which could be possible biological intermediates also.

iii) A new route of synthesis of coprostan-3α, 7α-diol from cholesterol which proceeds through the possible biological intermediates has been developed; cholest-4-en-3-one-7α-ol and coprostan-3-one-7α-ol have been obtained as intermediates in this synthesis.

iv) Cholest-4-en-3α, 7α-diol, a possible intermediate, which has not been described before has been synthesized.

v) Coprostan-3α, 7α-diol-24-one, another possible intermediate which has not been described before, has been synthesized.

As a result of the study of the reactions involved in the above mentioned syntheses, information has been obtained on certain steroid chemical reactions.

i) Further proof is provided for the α-configuration of the epoxide ring in cholest-4-en-3-one-6α, 7α-epoxide.

ii) Potassium borohydride reduction of cholest-4-en-3-one-6α, 7α-epoxide at reduced temperatures has been shown to reduce the keto group selectively; a new compound, cholest-4-en-3α-ol-6α, 7α-epoxide, has been synthesized.

The method of fluorescent thin-layer chromatography which has been adapted for use in synthetic steroid chemistry in this work has proved to be of importance in different ways. It has been successfully used as an additional check on the purity of most of the steroids used in this work and for co-chromatography of unknown steroids with standard samples. This particular thin-layer technique has also been used in checking different eluates obtained from column chromatography and in following many reactions such as in time-course studies. Valuable information about the mechanism of certain reactions and specificities of certain reagents has been obtained by following the reactions by TLC.

When cholest-4-en-3-one-7α-ol was hydrogenated at room temperature (p. 115), the TLC at every hour showed several spots, one less polar than the starting material and two or three more polar. The less polar spot seemed to be diminishing as the reaction

proceeded, while the more polar spots increased. This led to the belief that the less polar product was possibly an intermediate, and due to its chromatographic behaviour and non-u.v. absorbing nature, it was suggested to be coprostan-3-one-7α-ol. That this compound was an intermediate and was indeed coprostan-3-one-7α-ol was shown by the synthesis of this compound from the same starting material by hydrogenation at -27°. Another example of obtaining such information was the potassium borohydride reduction of cholest-4-en-3-one-6α,7α-epoxide (p. 106) where, with the help of this technique it was shown that the complete reduction of the epoxide proceeded through an intermediate cholest-4-en-3α-ol-6α,7α-epoxide. These two examples also showed the respective specificities of the hydrogenation and potassium borohydride reduction reactions at reduced temperatures; hydrogenation at -27° reduces the double bond only while potassium borohydride at -5°

reduces the ketone in preference to the epoxide group. Several other examples where TLC has given additional information about the reactions have been described in the text.

However, by this technique of fluorescent TLC, all studies are qualitative and it does not give as much information about the reactions as would be available if such studies were quantitative.

Thus, in this work a method has been developed whereby using small amounts of the reactants a quantitative study of the reactions can be performed (p. 50). For this new method the fluorescent TLC has been coupled with radioactive counting and the reactions are performed using labelled substrates. This renders the method quantitative and gives considerable additional information such as reaction rates. It is also possible to establish the number and amount of intermediates (or side products) in a reaction at a certain time. The purity of labelled

compounds can be checked and it can also be used for the study of compounds which do not give colour reactions. In this work this technique has been successfully used for the study of photo-sensitized oxygenation reactions (pp. 68 & 73)and it has been shown that using small quantities of reactants, a significant amount of information can be obtained about the mechanism of the reaction.

This technique, however, has the disadvantage that substances must be eluted from the plates for scintillation counting. To avoid this procedure of elution, the use of a chromatogram-scanning device could be considered. This will no doubt be a quicker and easier method but could not be as efficient as the liquid scintillation counting system used in this work; the efficiency of certain known chromatogram-scanners is very low compared with the 80% and 40% efficiency obtained for C^{14} and H^{3} respectively on the liquid scintillation counting system. The latter system was thus preferred and has given good results.

This method of the study of steroid reactions seems to have great promise in synthetic organic chemistry. The classical methods of studying organic reactions are tedious and not so precise. They usually rely on some method of estimation (like gravimetry) of the product and need relatively large amounts of the reactants and the product has to be worked-up before it can be estimated. The method described in this work, on the other hand, does not require any other specific methods of estimation of the products and can be used for very small quantities of the reactants; the product does not have to be worked up and so losses are cut down to a minimum. This method is thus more precise and easier and can give an almost 'minute-to-minute' account of the reaction.

Since this method is quantitative it can also be used for kinetic studies. But, since the purpose of this work was the synthesis of possible physiological degradation products of cholesterol and the main

interest was to study the time courses of certain reactions and to find out about the side-products and/or intermediates, if any, these studies were kept limited to these problems and the results obtained were not interpreted mathematically for the kinetics of the reactions. The data obtained could, however, be used for such interpretations.

It has been pointed out in the introduction that in the degradation of cholesterol to bile acids the initial attack is at C_7 and this hydroxylation could proceed through a hydroperoxide intermediate. 7α-Hydroxylation of cholest-5-en-3β, 26-diol and 3β-hydroxy-chol-5-enic acid has been shown in biological systems. If these hydroxylations were brought about by the same mechanism then several 7α-hydroperoxy and the corresponding 7α-hydroxy compounds would be intermediate.

In this work a method has been developed whereby 7α-hydroxylated derivatives have been synthesized through an intermediate photo-sensitized oxygenation reaction. The syntheses proceed through hydroperoxide intermediates, thus perhaps mimicing the biological hydroxylations. This method of 7α-hydroxylation, besides proceeding through the possible biological intermediates, has the advantage that it can be applied to a number of steroids. Use has been made of the 5, 6-double bond which makes the allylic position 7 very reactive and the hydroperoxidation of such steroids being a stereospecific replacement reaction (Nickon and Bagli, 1961), the hydroperoxide group is introduced specifically into the 7α-position. The existing methods of 7α-hydroxylation through an allylic bromination or an allylic oxidation at C_7 with the subsequent hydrolysis of the bromo compound or the reduction of the 7-ketone, do not give good yields and also the hydroxyl at 3β-position

has to be protected. In the photo-sensitized oxygenations, on the other hand, the hydroxyls need not be protected since the oxygen attack is stereo-specific at the 7α-position and under these conditions the hydroxyls are not attacked. Therefore, this method of 7α-hydroxylation was applied to other hydroxylated 5,6-unsaturated steroids besides cholesterol. It has been successfully used for the synthesis of cholest-5-en-3β,7α-diol and cholest-5-en-3β,7α-26-triol through the respective hydroperoxide intermediates. Preliminary experiments with methyl-3β-hydroxy-chol-5-enate have shown that this compound can be photo-oxygenated to produce the corresponding 7α-hydroperoxy derivative which could be reduced to the 7α-hydroxy compound. This compound could not be isolated due to paucity of material.

The hydroperoxides are very unstable compounds and decompose very easily to give several products (Kornblum et al. 1951). Fortunately, the hydro-

peroxides synthesized in this work were found to be stable on TLC. Thus, it was thought that these compounds would be stable on 'deactivated' silicic acid (p. 45) because this preparation of silicic acid has almost the same 'activity' as that of silica gel on thin-layer. Chromatography of these hydroperoxides on this grade of silicic acid gave a pure product. This finding is of interest because the hydroperoxides have been thought to be unstable on adsorbents such as alumina or silicic acid and that is why all purifications of these compounds have usually been accomplished by crystallization procedures. The reduction of the hydroperoxides gave the corresponding hydroxy compounds.

Cholest-5-en-3β, 7α-diol was shown to be pure by TLC and comparison of the product with an authentic sample prepared by a published method (Henbest and Jones, 1948). The infrared absorption spectrum shows large hydroxyl peaks and unsaturation, and the two infrared spectra from the two samples are identical.

Cholest-5-en-3β, 7α, 26-triol shows large hydroxyl peaks and unsaturation in the infrared spectrum. An infrared spectroscopic examination (kindly done by Dr J. Page of Glaxo Laboratories) was found to be identical with the spectrum obtained on the Infracord. Recrystallization of the compound did not raise its melting point and it ran as a single spot on TLC.

As discussed earlier (p. 19), cholest-5-en-3β, 7α, 12α-triol is a possible intermediate. This compound can be synthesized from 12α-hydroxy-cholesterol (Danielsson, 1961e) using the above method.

It is known that the tertiary hydrogens in an organic molecule are more reactive than the secondary and primary hydrogens. cis-Decalin forms a hydroperoxide at one of the tertiary carbon atoms (C_9) replacing the hydrogen with a hydroperoxide group, showing that hydroperoxidation can occur on a

saturated tertiary carbon atom. The cholesterol molecule has several such reactive centres and if the biological hydroxylations are mediated through hydroperoxide intermediates, it would seem reasonable to assume that these positions will be most prone to attack. Of all the tertiary carbon atoms in the cholesterol molecule, C_{25} is of interest in the study of degradation of cholesterol to bile acids (p. 38). As has been discussed earlier, the C_7 is a very reactive centre and it is not surprising that the initial attack may be at that position. Once the C_7 has been attacked to form a hydroxyl, and if the other hydroxylations occur through hydroperoxides, then an attack at the tertiary carbon C_{25} would be most likely. Cholest-5-en-3β, 25-diol has been isolated in in vitro studies (Fredrickson, 1956). This, however, does not disprove that a 25-hydroxylation is one of the stages of degradation of the cholesterol side-chain. It may be that 7α-hydroxylation has to precede

the 25-hydroxylation and in such a case cholest-5-en-3β, 7α, 25-triol would be an intermediate. This compound can be synthesized from cholest-5-en-3β, 25-diol by the above method of 7α-hydroxylation. Cholest-5-en-3β, 7α, 24-triol, which is another possible intermediate (p. 25), can be synthesized from cholest-5-en-3β, 24-diol. These two starting diols, cholest-5-en-3β, 24-diol and cholest-5-en-3β, 24β-diol have been synthesized by known methods discussed later.

The sequence of reactions used in this work for the synthesis of coprostan-3α, 7α-diol from cholesterol (p. 100) is based on the fact that the secondary 3β-hydroxyl of cholesterol is in a homo-allylic position to the 5, 6-double bond and so can be oxidized by an Oppenauer method. The other hydroxyls, if any, in the molecule are not affected. The epoxidation of the product of the Oppenauer oxidation, cholest-4, 6-diene-3-one, by mono-perphthalic acid is also a selective oxidation for this double bond (Δ^6). Several reactions are known where

monoperphthalic acid has been used for selective epoxidation of double bonds in steroids having hydroxyl functions or ester groups in the molecule (for references see Fieser and Fieser, 1959). The reduction of the epoxide with lithium aluminium hydride does not affect any hydroxyls and the reoxidation of the reduced product with manganese dioxide is again a selective oxidation for the hydroxyls allylic to a double bond. The formation of coprostan-3-one-7α-ol by selective reduction of the double bond in cholest-4-en-3-one-7α-ol at reduced temperatures does not affect any hydroxyl groups. The carbonyl of the coprostan-3-one-7α-ol has been reduced with metal hydrides to give coprostan-3α, 7α-diol.

All the reactions described are selective and would leave the other hydroxyls, if any, untouched and thus the method can be applied to other hydroxylated Δ^5-steroids. The methods of synthesis of cholest-5-en-3β, 26-diol (Scheer et al. 1956), cholest-5-en-3β, 12α-diol (Danielsson, 1961e), cholest-5-en-3β, 25-diol (Dauben et

al. 1950; Ryer et al. 1950) and cholest-5-en-3β, 24-diol (Riegel and Kaye, 1944) are known, and using these substances as the starting materials, a whole series of possible intermediates can be synthesized. Some such possible intermediates are listed on pages 19 and 29.

Nickon and Bagli (1961) first prepared cholest-4-en-3-one-6α, 7α-epoxide and indicated that the α-configuration of the epoxide was arbitrary and was based on analogy with known reactions (Nussbaum et al. 1958). The synthesis of cholest-4-en-3-one-7α-ol and some of its derivatives from this epoxide has lent a direct proof for the α-configuration of the epoxide group. The production of cholest-4-en-3-one-7α-ol also shows that the reductive cleavage of the epoxide ring with lithium aluminium hydride does not proceed with an inversion like in some cyclohexane epoxides (Trevoy and Brown, 1949). The reason for this seems to be the fact that in cyclohexane the ring is free while the ring B of steroids is rigidly fixed and the reductive fission does not proceed with inversion.

Studies on metal hydride reductions of cholest-4-en-3-one-6α,7α-epoxide have shown that the reaction was faster with lithium aluminium hydride and resulted in a complete reduction to the two isomeric diols. The reduction with sodium or potassium borohydride, on the other hand, proceeded much more slowly, and the epoxide was not reduced completely; studies using thin-layer chromatography showed that the reaction proceeded through the intermediate formation of a compound more polar than the starting epoxide and less polar than the diols. Its chromatographic behaviour suggested that it had one nuclear hydroxyl with some other less polar substituent; it was not u.v. absorbing and gave a light brown colour with phosphotungstic acid. The reduction of the epoxide with potassium borohydride at reduced temperature (-5°) gave this compound in good yields. Its infrared spectrum does not show a peak characteristic of ketones but gives a hydroxyl peak (Fig. 7 Appendix) and it is not u.v. absorbing which shows that the 3-ketone has been reduced. Manganese dioxide oxidation gives

the starting epoxide showing the allylic unsaturation at 4, 5 and the presence of the epoxide group. The 3α-configuration of the hydroxyl is proved by treatment with digitonin which did not precipitate the compound. This compound when reduced with lithium aluminium hydride gave the diol cholest-4-en-3α, 7α-diol. This proves that the compound is cholest-4-en-3α-ol-6α, 7α-epoxide and shows that potassium borohydride reduces the ketone in preference to the epoxide group and also that the reduction with this reagent gives the 3α-isomer as the major product. The reduction of 3-ketones with a bulky hydride reagent would be expected to give the equatorial 3β-isomer as the major product. McKennis and Gaffney (1948) and Plattner et al. (1948) showed that the reduction of steroid Δ^4-3-ketones with lithium aluminium hydride gave a 1:1 mixture of the two 3α- and 3β-hydroxy isomers. In the case of the reduction of cholest-4-en-3-one-6α, 7α-epoxide it has been shown in this work that the lithium aluminium hydride reduction

gives a mixture of diols but potassium borohydride gives the axial 3α-isomer. The reason for this seems to be that the epoxide group at the rear of the molecule prevents an attack of the metal hydride reagent from that side and the attack is frontal to give the 3α-isomer. The selectivity of this attack is increased when lithium aluminium hydride is replaced with a bulkier reagent like potassium borohydride.

Cholest-4-en-3α,7α-diol, a possible intermediate in the degradation of cholesterol (p. 17) has not been described before. This compound has been synthesized and its structure shown by different methods. The infrared absorption spectrum shows large hydroxyl peaks and unsaturation. Manganese dioxide oxidation gave a known compound, cholest-4-en-3-one-7α-ol, proving the presence of a hydroxyl allylic to a 4,5-double bond and a hydroxyl in the 7α-position. The 3α-configuration of the hydroxyl is proved by non-

precipitation of an alcoholic solution of the compound with digitonin. This compound would be expected to give coprostan-3α, 7α-diol on saturation; the hydroxyls in the 3α- and 7α-positions would hinder an attack from the α-side and the reduction of the double bond would give a 5β-hydrogen giving coprostan-3α, 7α-diol as the product. It will be interesting to study the possible conversion of this compound to bile acids in bile fistula animals.

Cholest-4-en-3-one-7α-ol has been synthesized by a new route. While this work was in progress, Danielsson (1961a) published a method of synthesis of this compound but the method developed in this work gives much better yields and is applicable to other hydroxylated steroids also. As discussed on page 100, 26, 25, 24 and 12α-hydroxylated derivatives of cholest-4-en-3-one-7α-ol can possibly be synthesized using the corresponding dihydroxy derivatives of cholesterol as the starting materials. The compound has the same

characteristics as the published data (Danielsson, 1961a). Its structure is further proved by the synthesis of derivatives like coprostan-3-one-7α-ol and coprostan-3α, 7α-diol. These derivatives had the same characteristics as the published data.

It has been discussed in the Introduction that coprostan-3-one-7α-ol and coprostan-3α, 7α-diol are possible intermediates in the degradation of cholesterol to bile acids.

Cholest-4-en-3-one-7α-ol would be expected to give coprostan-3α, 7α-diol on catalytic hydrogenation. With the help of TLC in this work it was shown that this reaction proceeded through the intermediate formation of coprostan-3-one-7α-ol. This finding was used in the selective reduction of the 4, 5-double bond of cholest-4-en-3-one-7α-ol at -27° (cf. Grasshof, 1934; Nickon and Bagli, 1961) to produce coprostan-3-one-7α-ol. This compound was first prepared by Yamasaki et al. (1959a). Danielsson (1961b) published another

synthesis of this compound but both these methods do not give good yields and the starting material, coprostan-3α, 7α-diol itself is difficult to synthesize. The advantage of the synthesis described in this work is also that the starting material is cholesterol itself and the synthesis proceeds through the possible biological intermediate cholest-4-en-3-one-7α-ol. The structure of this compound, coprostan-3-one-7α-ol, has been shown by comparison of the properties of the compound with the published data. TLC gives one spot whose mobility suggests the proposed structure; infra-red absorption spectrum shows hydroxyl and carbonyl absorption; it forms coprostan-3α, 7α-diol on reduction with lithium aluminium hydride.

Coprostan-3α, 7α-diol, another proposed intermediate, has been synthesized by different methods. Catalytic hydrogenation of cholest-4-en-3-one-7α-ol or cholest-4-en-3-one-6α, 7α-epoxide at room temperature produced coprostan-3α, 7α-diol. The synthesis was

found to be more effective if the starting material was hydrogenated first at -27° and then at room temperature. This seems to be due to the fact that at room temperature the hydrogenation may reduce the 3-ketone before the saturation of the double bond and in such a case two isomeric products (3α-ol and 3β-ol) would be obtained from the 3-ketone. But if the reaction is conducted first at -27°, only the double bond is reduced resulting in a saturated derivative, of the coprostane series, the reduction of which will mainly give the 3α-isomer since it is known that the reduction of 3-ketones of the coprostane series gives mainly the 3α-isomer.

Coprostan-3α, 7α-diol was also obtained when coprostan-3-one-7α-ol was reduced with lithium aluminium hydride. Another new method of synthesis was by a Huang-Minlon reduction of coprostan-3α, 7α-diol-24-one which is a new compound synthesized in this work.

That the compounds obtained by the four methods of synthesis were identical was shown by mixed melting point determinations and comparison of the infrared spectra; all infrared spectra are identical and show large hydroxyls and no unsaturation. The samples were also compared with an authentic sample of coprostan-3α, 7α-diol prepared by electrolytic coupling of chenodeoxycholic acid and iso-valeric acid (Bergstrom and Krabisch, 1957).

Bergstrom and Krabisch (1957) and Yamasaki et al. (1959) noticed that this compound did not crystallize very well. In this work it was noticed that the product obtained from the hydrogenation reactions when evaporated gave a solid product having a higher melting point (188-192°) but when it was crystallized from any solvent the melting point was much lower (78-79°). As Bergstrom and Krabisch pointed out, this lowering of the melting point may be due to the molecule of water of crystallization. The synthesis of this

compound from cholesterol via cholest-4-en-3-one-7α-ol and coprostan-3-one-7α-ol is of interest since it proceeds through the same intermediates as have been proposed for the biosynthesis of this compound.

Several methods have been used to prepare some possible intermediates with modifications in the side chain. 26-, 25- and 24-hydroxylated derivatives of cholesterol have been synthesized by known methods. These compounds could serve as the starting materials for the synthesis of a whole series of compounds by the two methods described in this work. By the method of 7α-hydroxylation the three triols can be synthesized and these compounds can also be used for the synthesis of 12α, 26-, 25- and 24-hydroxylated intermediates connected with the inversion of configuration at C_3 and saturation of the double bond (pp. 15 and 99).

Cholest-5-en-3β, 26-diol was prepared from the plant sterol kryptogenin. Scheer et al. (1956) prepared this compound from the same sterol through a

Clemmensen reduction and a subsequent Huang-Minlon reduction of the product of this reduction. This second reduction was found necessary because the 16-carbonyl of kryptogenin is not very easy to reduce presumably because the position-16 is shielded by the side-chain and so is difficult to attack by the reducing reagents. The 22-carbonyl on the other hand does not produce such a problem. In this work the Clemmensen reduction of kryptogenin was studied with the help of TLC and it was noticed that if the reaction was carried out for a longer time (up to $2\frac{1}{2}$ hours), most of the 16-carbonyl was also reduced. If the reaction was kept on longer than 3 hours, some additional products were produced. Thus 2 hours was taken as the standard time and the product was worked up at that stage. It was found that the isolation of the 26-hydroxycholesterol from the product mixture was quite easy through the chromatography of the acetylation product of the

mixture. However, the chromatography of the diacetates on alumina produced an interesting observation; the diacetates seemed to undergo a partial hydrolysis of the primary alcoholic acetate group at C_{26}. Although most of the product still remained as the diacetate and the separation was quite effective, about 12% of the product underwent a partial hydrolysis and about 5% showed complete hydrolysis. This latter observation does not agree with the findings of Johns and Jerina (1963) who indicated that the steroid primary alcoholic acetates got hydrolysed while the secondary did not. The free cholest-5-en-3β, 26-diol was obtained by the hydrolysis of the diacetate. This compound has been used for the synthesis of cholest-5-en-3β, 7α, 26-triol prepared by the method of 7α-hydroxylation developed in this work.

Cholest-5-en-3β, 25-diol has been obtained from 25-keto-nor-cholest-5-en-3β-ol. The product was obtained in better yields when the purification was done

by chromatography. This compound was synthesized because it can serve as a starting material for the synthesis of other 25-hydroxylated intermediates using the two methods developed in this work. This compound is also of interest because this can be a precursor of cholest-5, 24-dien-3β-ol (Desmosterol) which has been shown to be a direct precursor of bile acids. As discussed on page 38, there seems to be a possibility that 25-hydroxylation may occur after 7α-hydroxylation. Danielsson and Johansson (1964) using Desmosterol as the substrate in their in vitro experiments with the mouse liver homogenate have reported the formation of a compound, whose properties suggested it to be cholest-5, 24-diene-3β, 26-diol. This is possible because the double bond at C_{24} would render the allylic position 26 very reactive for a hydroxylation reaction. The hypothesis that Desmosterol is a precursor of cholesterol has recently been questioned (Goodman et al. 1963). In

view of the above observations it would be reasonable to postulate that a 25-hydroxylation may be the first stage of degradation of 7α-hydroxycholesterol and this intermediate, cholest-5-en-3β, 7α, 25-triol could dehydrate to give cholest-5, 24-diene-3β-ol which would then hydroxylate in the allylic position C_{24}.

Cholest-5-en-3β, 24-diol was prepared by two methods. The known method of synthesis from 3β-hydroxy-chol-5-enic acid is tedious because of the difficulties involved in preparing the starting material. The synthesis proceeds through several stages; purification and crystallization at every stage was found to be quite difficult. Thus another method was tried and the compound was obtained relatively easily by ozonolysis of fucosterol and the subsequent reduction of the 24-ketone with potassium borohydride. These two samples were compared with an authentic sample of cholest-5-en-3β, 24β-diol supplied by Professor A. Ercoli.

The structure and purity of these three diols (26, 25, 24) and the intermediates in their synthesis have been shown by comparison with the published data, their infrared spectra and behaviour on TLC - they all gave single spots.

Cole and Julian (1945) found a method of preparing steroids with a ketone in the side-chain. A new compound, coprostan-3α, 7α-diol-24-one, has been synthesized by this method (p. 155). The compound could not be brought into a crystalline form although several different solvents were tried for this purpose. Evaporation of an ether solution of the compound, however, gave a colourless powder which ran as a single spot on TLC in two different solvent systems. The structure of the compound has been shown by the usual methods. TLC behaviour suggests the presence of two hydroxyl groups and a less polar substituent; it is less polar than the 3α,7α, 12α-trihydroxy-coprostan and more polar than 3α, 7α-dihydroxy-coprostan (solvent system - benzene:ethyl acetate: acetone, 10:5:3). Its infrared spectrum shows a large hydroxyl peak and a carbonyl absorption.

This compound is of interest in this work because this could give some information about the mechanism of the cleavage of the cholesterol side-chain. It has been pointed out in the Introduction (p. 25) that in the cleavage of the side-chain, 24-hydroxylation may be involved and thus coprostan-3α, 7α, 24-triol may be a possible intermediate. However, experiments using compounds carrying a C_{24}-oxygen function have shown that these compounds are indeed converted into cholic acid in bile fistula animals, but not as readily as the corresponding compounds without a C_{24}-oxygen. Such studies have been performed on 3α, 7α, 12α-trihydroxy coprostanic compounds with or without a C_{24}-oxygen. The same studies using compounds not already carrying a 12α-hydroxyl group will be of greater significance since they can give information on two important points.

It is known that compounds carrying an oxygen function (hydroxyl or carboxyl) at C_{26} do not produce

cholic acid indicating that the 12α-hydroxylation does not occur once the oxidation of the side-chain has started. Such studies have not been done on compounds carrying a C_{24}-oxygen (hydroxyl or carbonyl) and no 12α-hydroxyl. The results with the latter compounds may be quite different. Thus it will be of interest to ascertain (a) if the coprostan-3α, 7α-diol-24-one or the corresponding coprostan-3α, 7α, 24-triol are at all converted into cholic acid and (b) whether they are converted into chenodeoxycholic acid more or less readily than coprostan-3α, 7α-diol or coprostan-3α, 7α, 26-triol.

It will be interesting to perform the same sort of comparative studies using cholesterol, 24-hydroxycholesterol and 26-hydroxycholesterol.

Cholesterol is present in all mammalian cells, in membrane and in sub-cellular particles in relatively large amounts. It appears to be present among the most primitive animals but only as one of several sterols. In higher animals the proportion of cholesterol to other sterols increases until it becomes the principal sterol of the vertebrates. In the higher animals the sterols, principally cholesterol, occur as free alcohols or esterified with carboxylic acids and also associated with other substances such as proteins to give lipoprotein complexes. The sterols are continuously formed and metabolized to other products in the animal and plant cells.

Cholesterol itself is quite inert until it is activated. This activation could occur in different tissues at different particular positions of the cholesterol molecule leading to the formation of a variety of metabolites. If the activation occurs in the liver, the molecule degrades in a particular manner to form bile acids and

neutral sterols while if the activation occurs in endocrine tissue the products are steroid hormones. Activation in plants may lead to different types of sterol; in the lower plants the molecule can add on one or two carbon atoms at C_{24} such as in fucosterol, but in other plants activated at 16, 22 and 26 positions it could form oxygenated derivatives which give a number of other derivatives by dehydration and saturation. The plant sterols with rings E and F could arise in this manner.

Thus, in short, the chemistry of cholesterol and related sterols is of great importance in different fields of study. In all the biological processes described in the preceding paragraph, the oxygenations or hydroxylations of the cholesterol molecule seem to be very important reactions. As discussed in the review of the subject (p. 31), there seems to be a possibility that these hydroxylations are brought about through hydroperoxide intermediates but there is no direct experimental evidence to back this hypothesis.

The work described here has been undertaken on a minor problem, viz. studies on some physiological degradation products of cholesterol in the formation of bile acids. As has been discussed in the text, there are a number of possible routes for this degradation and it is not known which of them is actually employed in the biological processes. The sequence of the degradative steps is not known and there is little information available on the actual mechanism of these reactions, for example the mechanism of hydroxylations is not clear.

In this work an effort has been made to review the subject and show that there are still 'gaps' in our knowledge of these processes.

The studies on these biological processes have usually been performed through synthesized 'possible intermediates'. Thus in this work a number of such compounds which could be postulated as intermediates in this degradation have been synthesized by different methods:-

Hydroxylation at C_7 of the cholesterol molecule has been achieved by two different methods - viz. the photo-sensitized oxygenations, through a hydroperoxide intermediate, and with per-acids through an epoxide intermediate. It has been shown that this method of hydroxylation can be applied to a number of Δ^5-steroids and a whole series of possible intermediates can be synthesized.

A new route for the synthesis of coprostan-3α, 7α-diol has been evolved which proceeds through the possible biological intermediates cholest-4-en-3-one-7α-ol, and coprostan-3-one-7α-ol. This method of synthesis is also applicable to a number of Δ^5-steroids having other hydroxyls in the molecule and using this method series of possible intermediates can be synthesized.

Cholest-4-en-3α, 7α-diol, another possible intermediate, not described before, has been synthesized.

Another new compound, cholest-4-en-3α-ol-6α, 7α-epoxide is described.

A synthesis of coprostan-3α, 7α-diol-24-one is described. This compound could be of importance in the study of the mechanism of the cleavage of the cholesterol side-chain.

Fluorescent thin-layer chromatography has been used for following most of the reactions. A method has been developed whereby steroid reactions could be studied quantitatively with the help of fluorescent thin-layer chromatography and scintillation counting.

The Wilzbach method of tritium-labelling has been improved by giving the compound a shorter exposure with tritium; the purifications have been effected by chromatography.

SECTION VII

APPENDIX

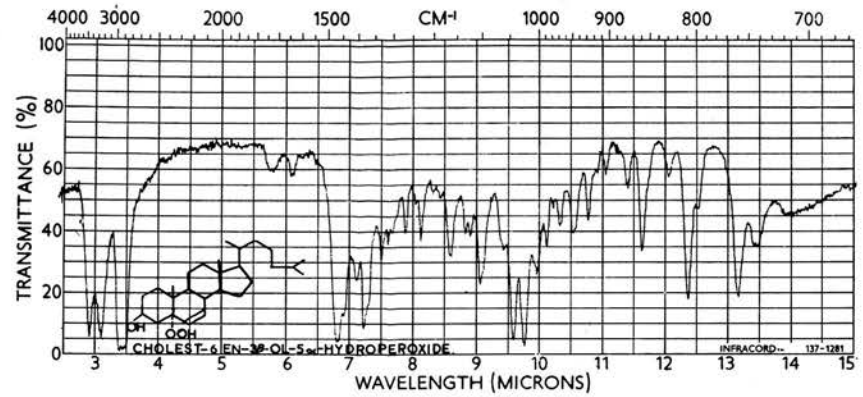


Figure 1

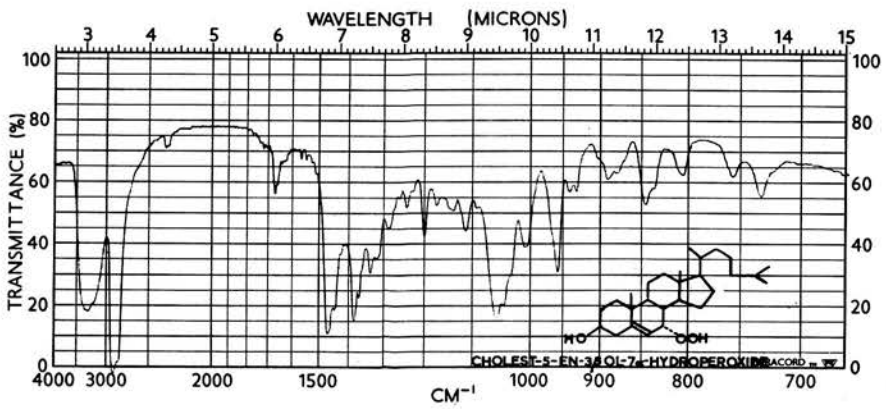


Figure 2

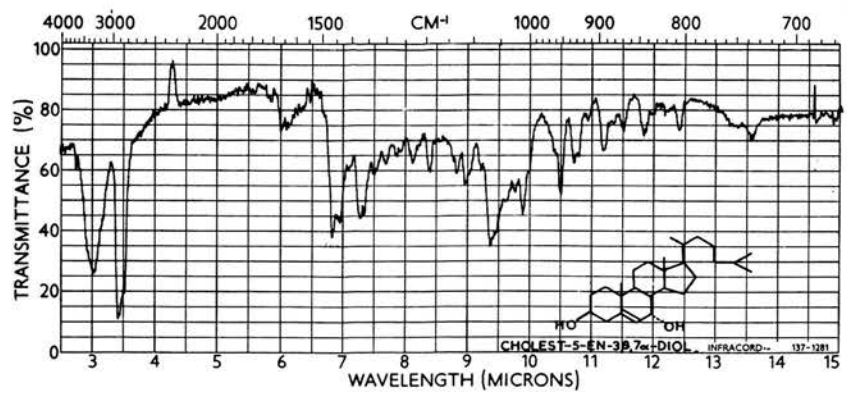
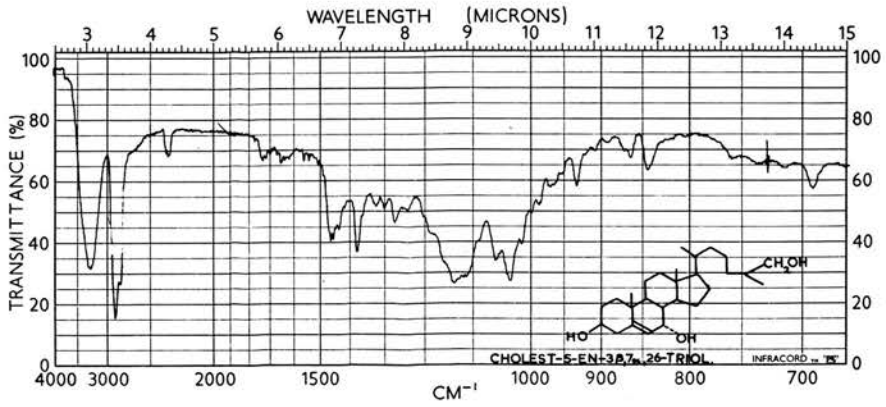


Figure 3



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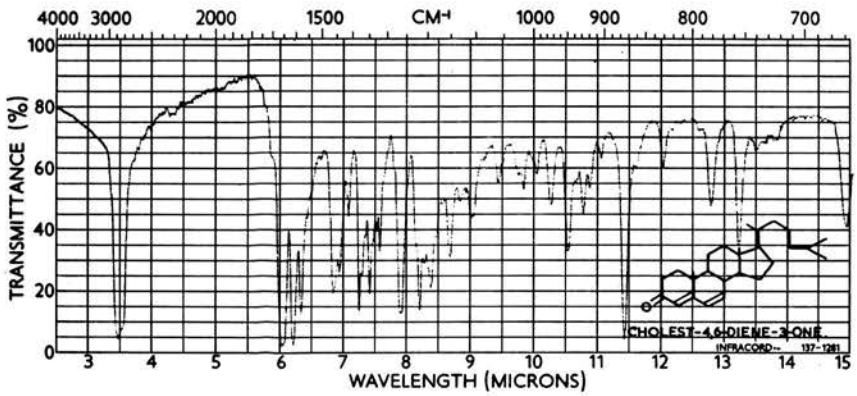
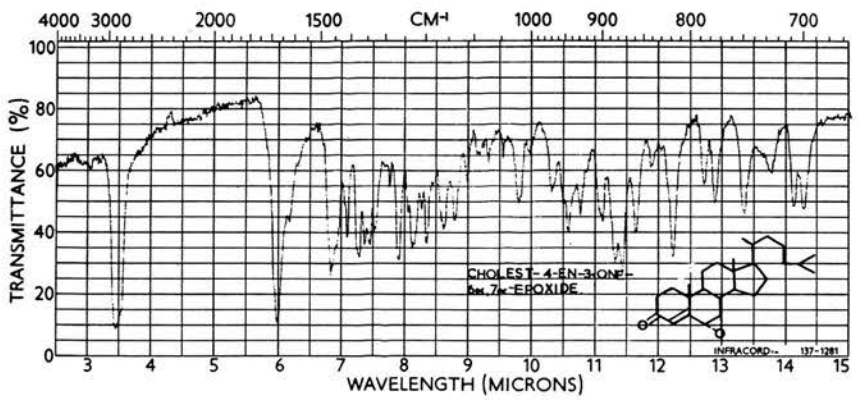


Figure 5



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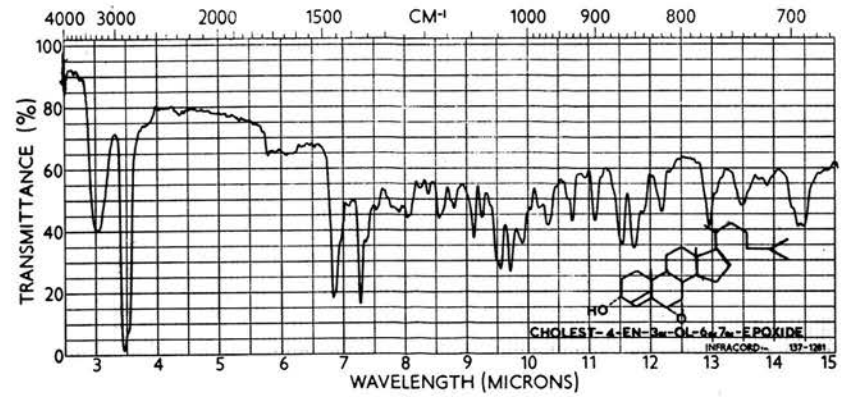

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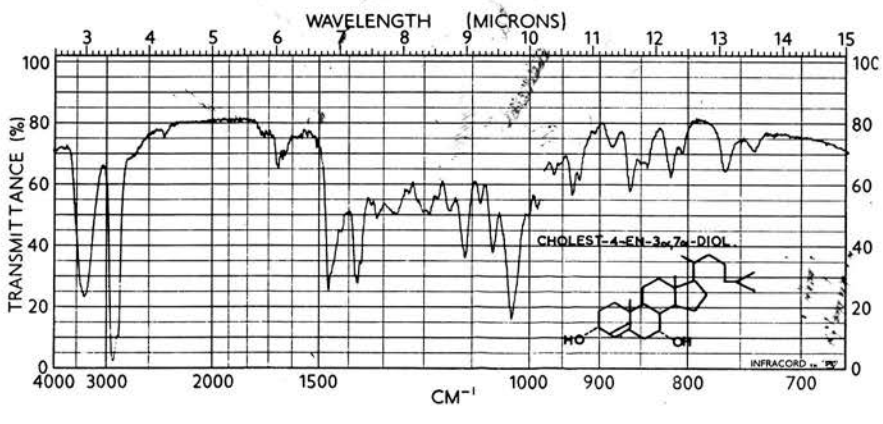
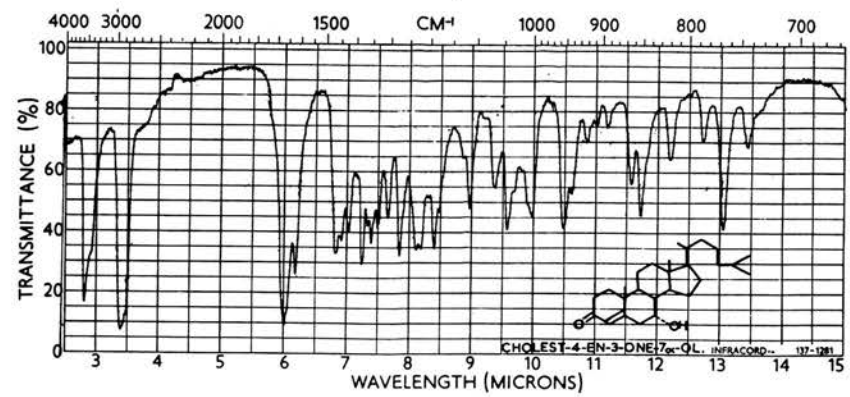


Figure 8



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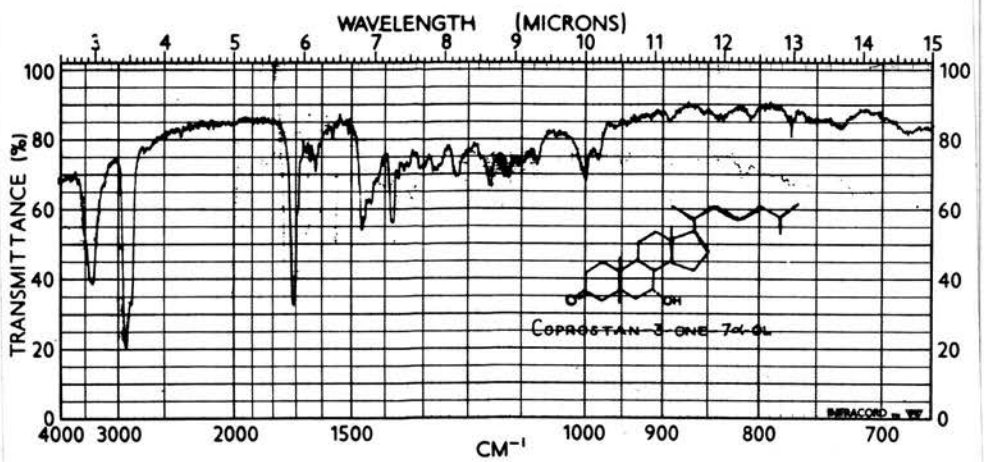

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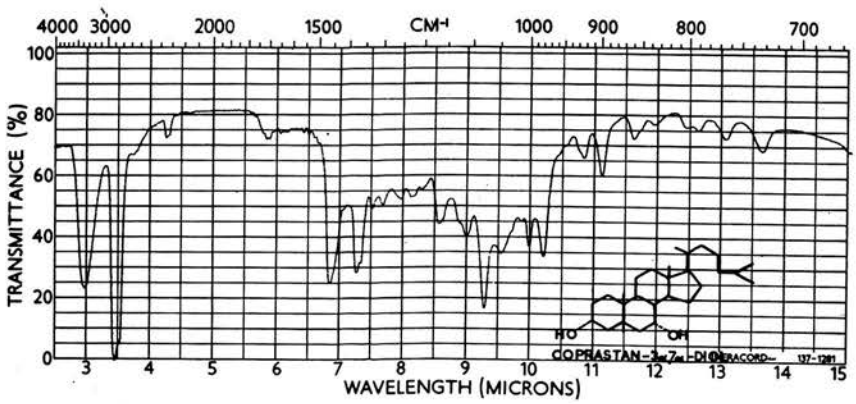
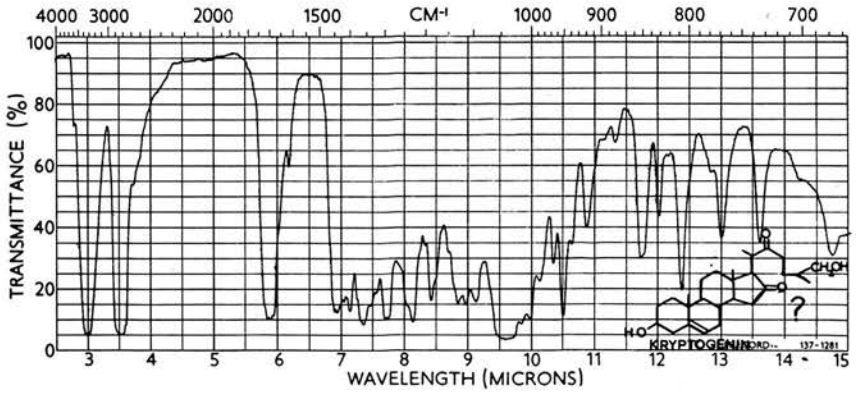


Figure 11

Figure 12



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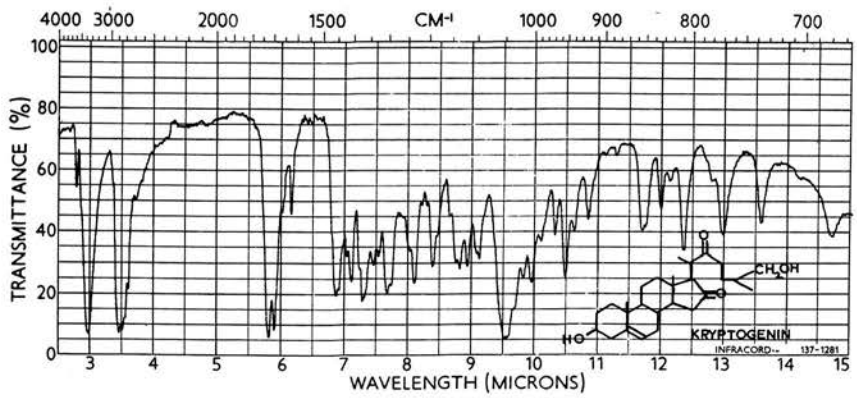

Figure 14

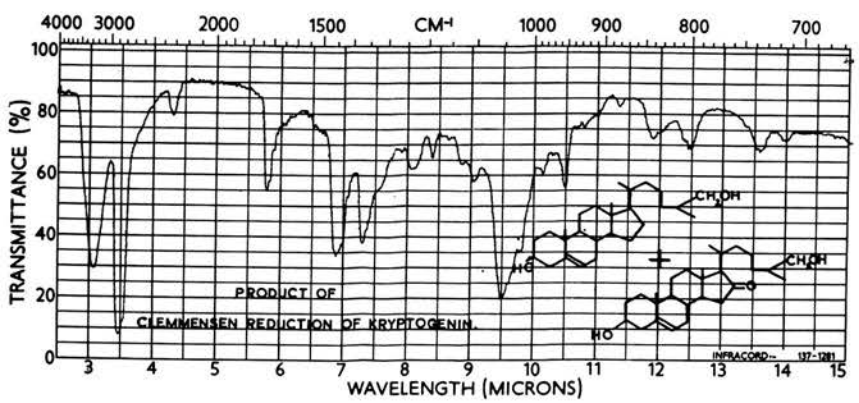

Figure 15

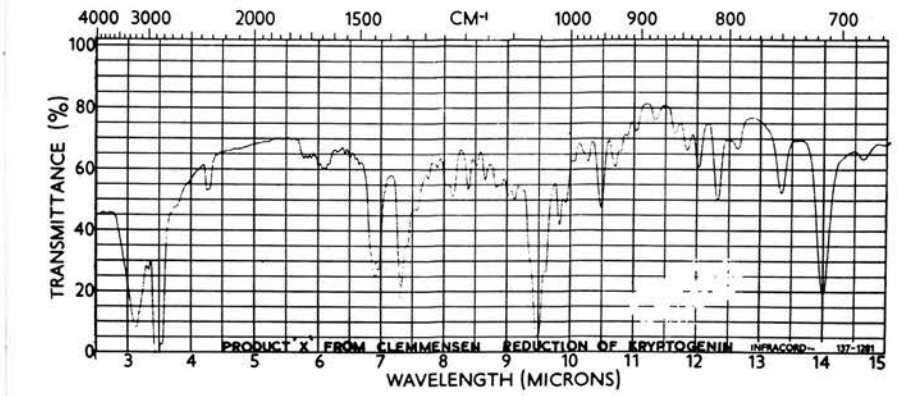

Figure 16

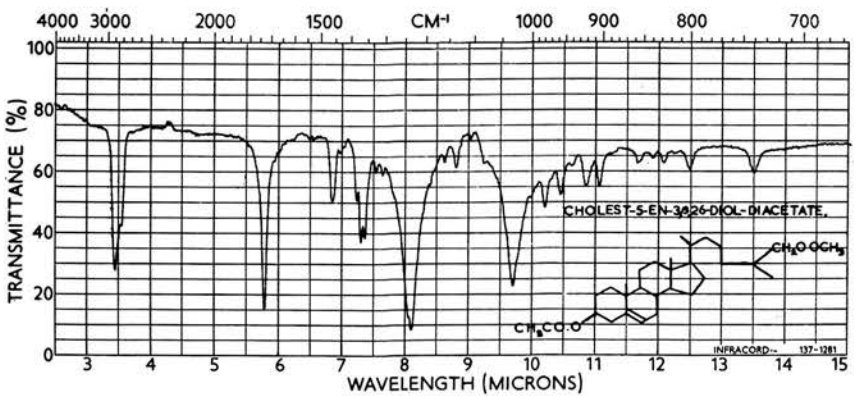

Figure 17

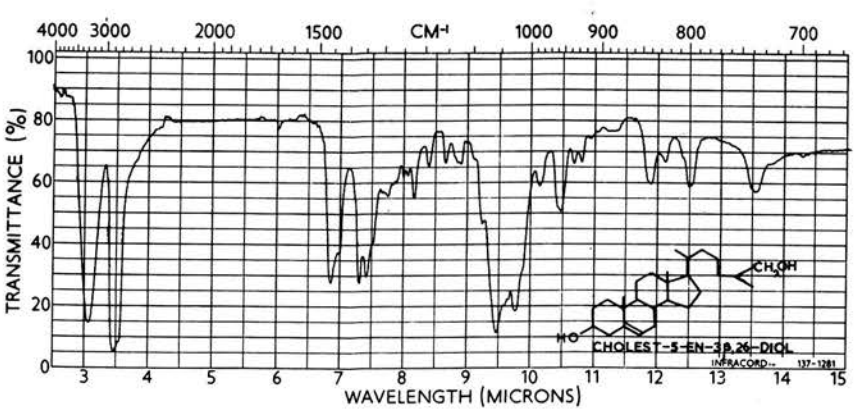

Figure 18

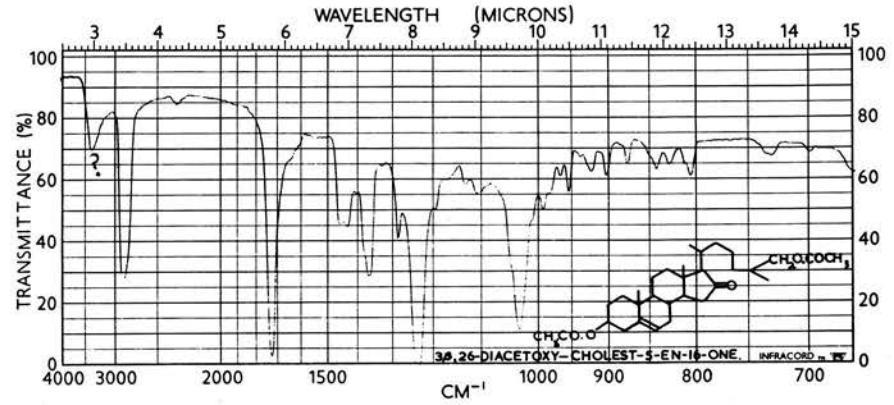

Figure 19

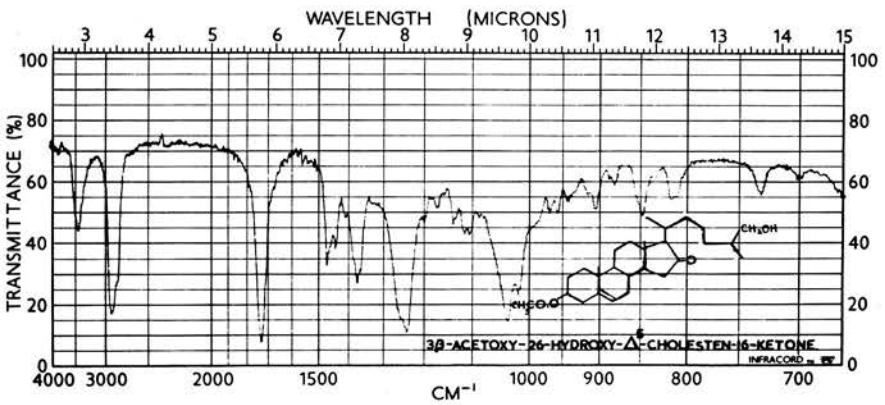

Figure 20

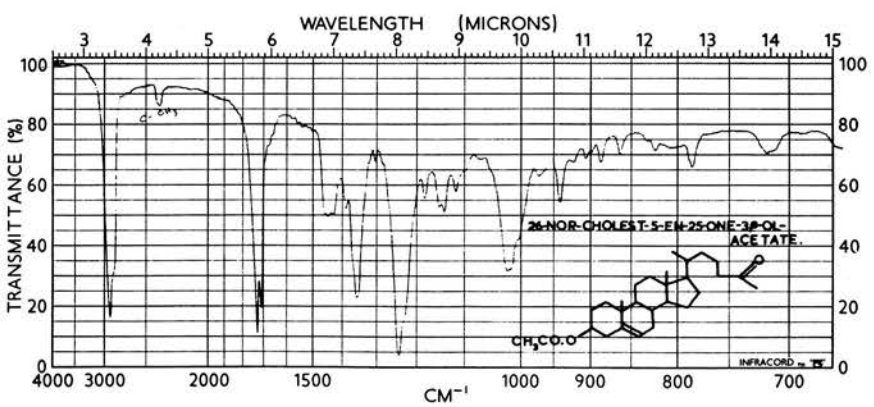

Figure 21

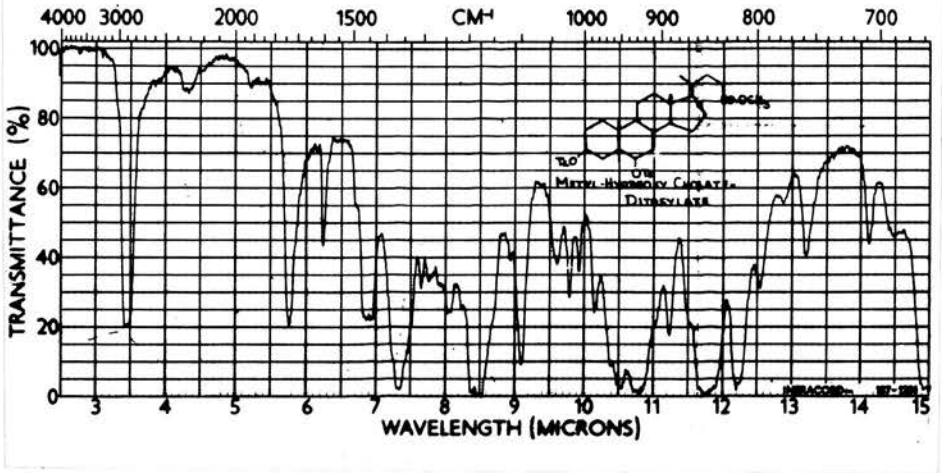

Figure 23

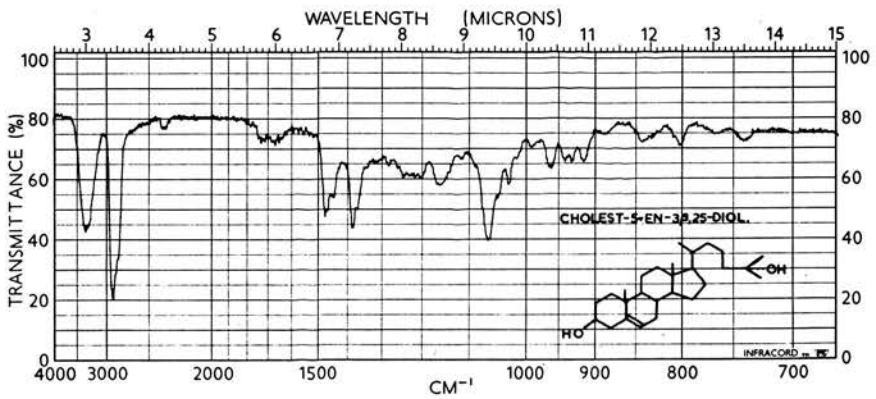

Figure 22

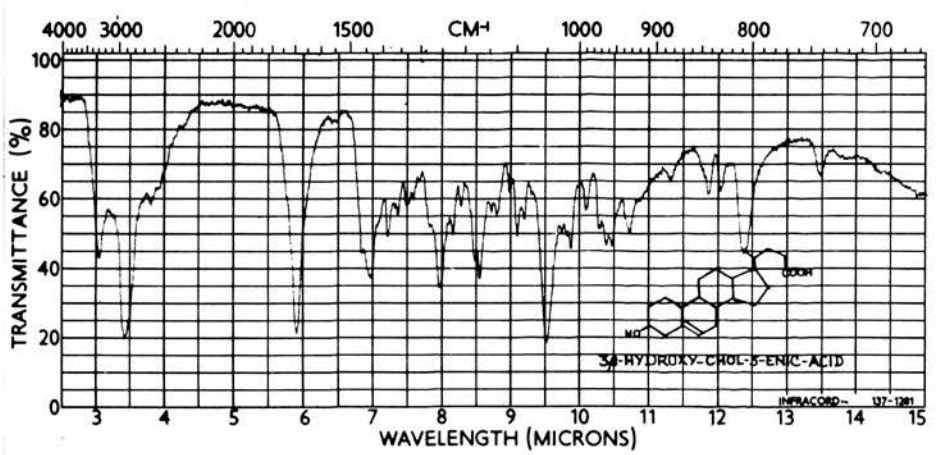

Figure 24

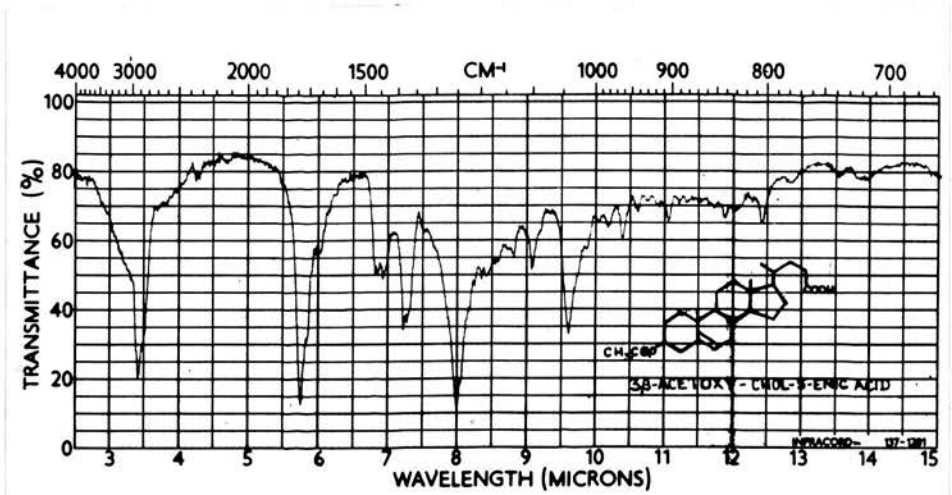

Figure 25

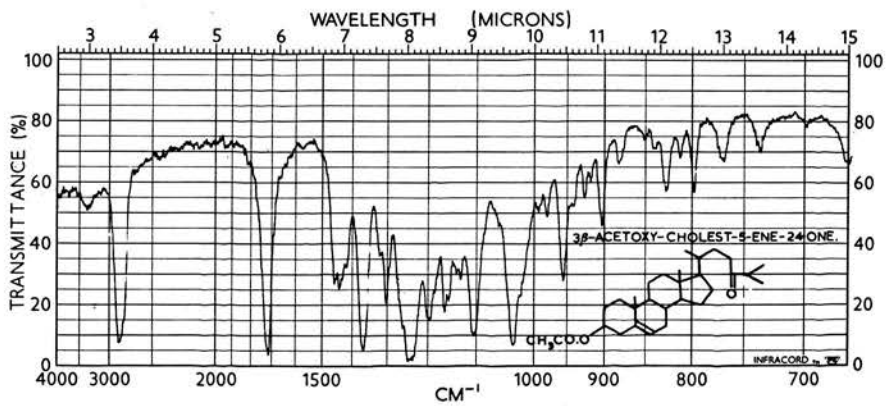

Figure 26

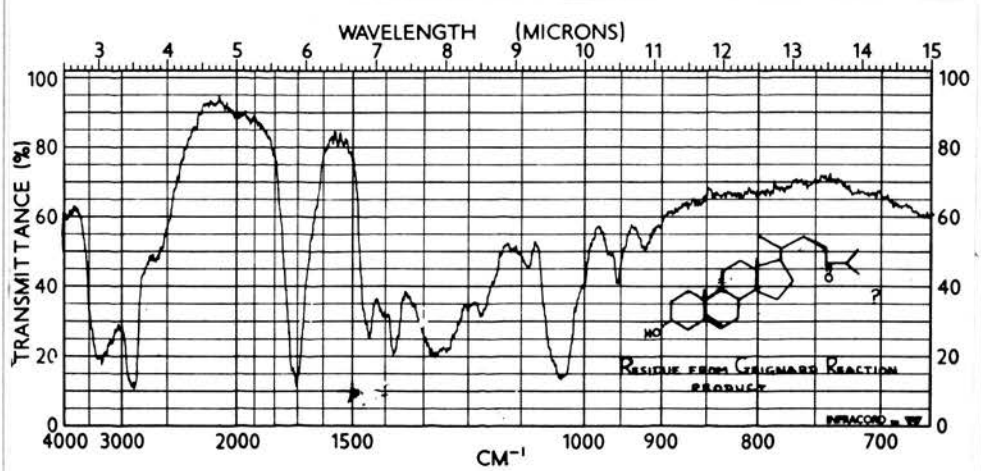

Figure 27

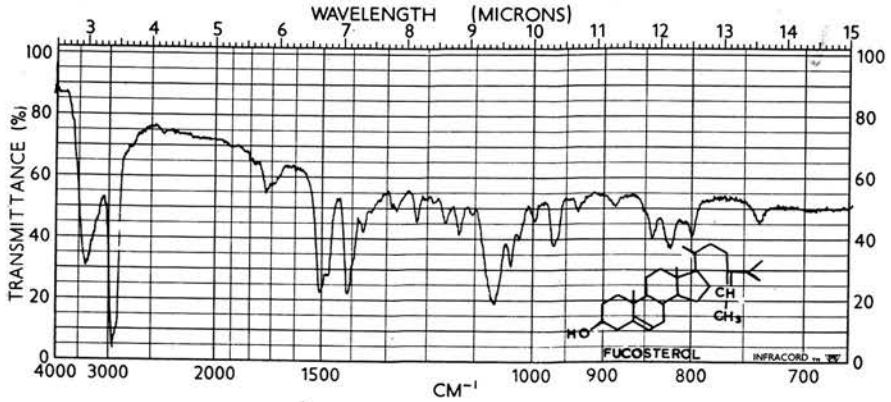

Figure 28

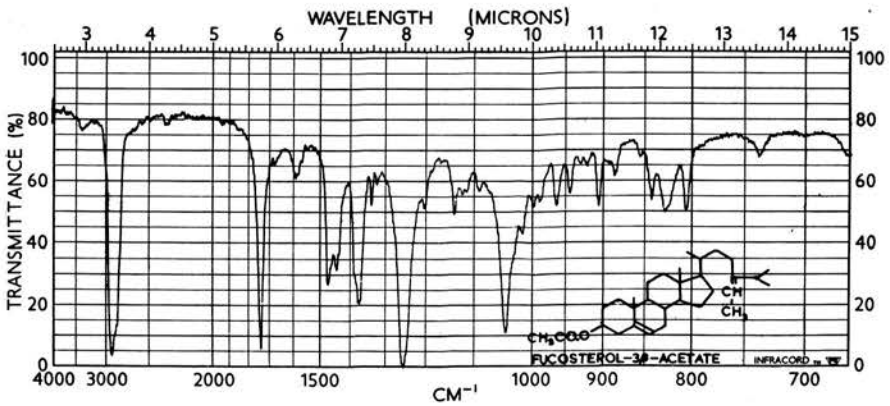

Figure 29

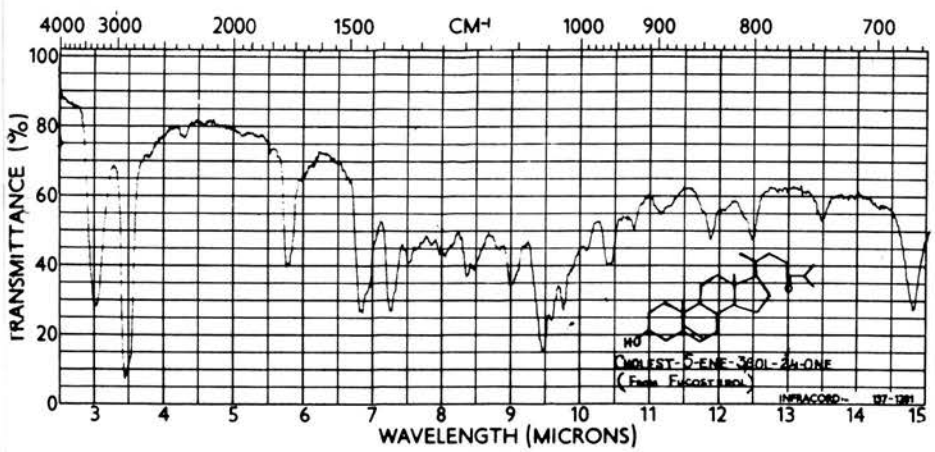

Figure 30

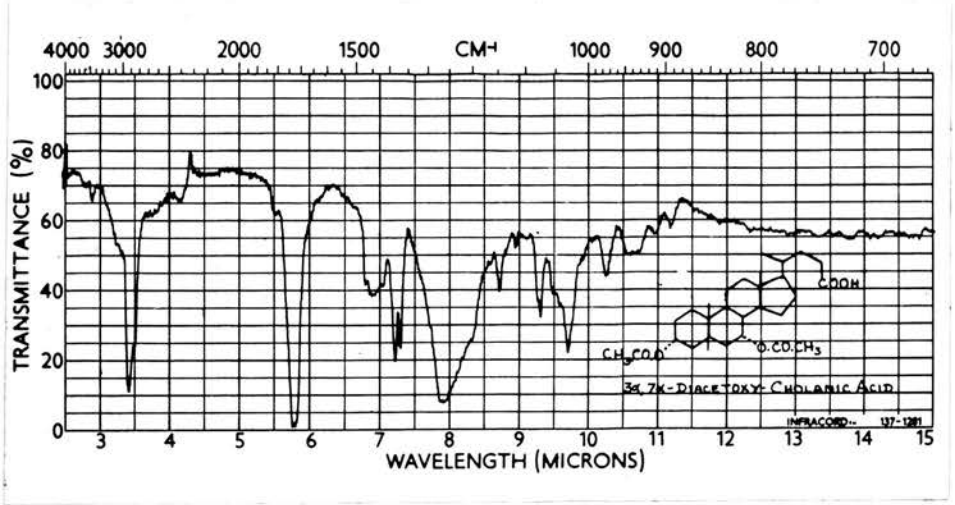

Figure 31

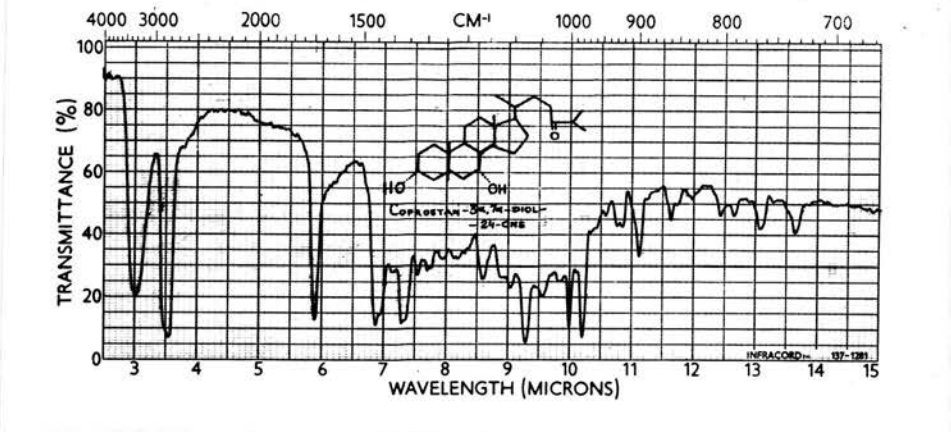

Figure 32

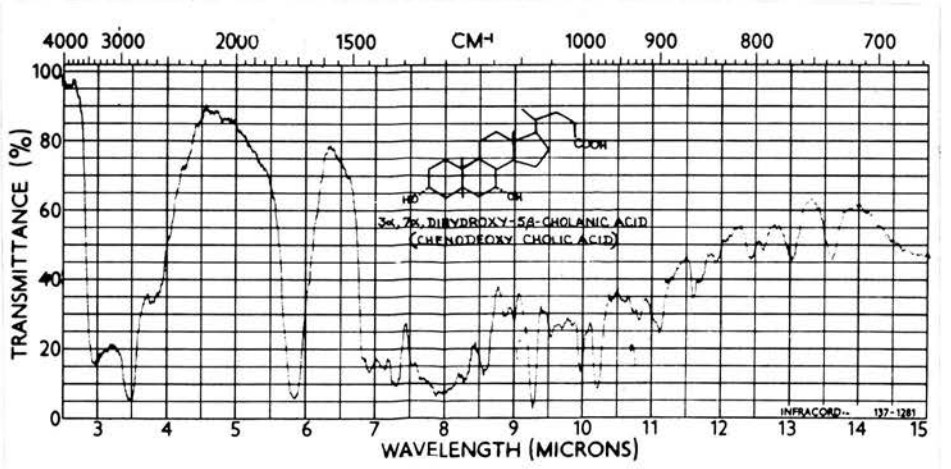

Figure 33

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